Biodegradation of Organic Contaminants in Subsurface Systems: Kinetic and Metabolic Considerations

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(ABSTRACT)

Groundwater contaminated by organic chemicals from industrial spills, leaking underground gasoline storage tanks and landfills has caused concern about the future of a major source of drinking water. Compounds from industrial sources such as alcohols and phenols are frequently found as groundwater contaminants. These compounds are highly soluble in water and do not adsorb well to aquifer material. They also have the potential to migrate in the subsurface system achieving significant levels in drinking water supplies. In addition, they can serve as carriers for carcinogenic compounds such as benzene, toluene and xylene which are relatively insoluble in water, but are quite soluble in alcohol.

A potential alternative to expensive groundwater reclamation projects is the use of the natural soil bacteria to degrade organic contaminants. Very little is known, however, about the response of subsurface soil bacteria to man-made organic chemicals or the degradation rates of these compounds. Such information would be useful in planning cleanup or protection strategies for groundwater systems. This study was designed to measure the kinetic response of tertiary butyl alcohol (TBA), determine the biological degradation rates of methanol, ethanol, propanol, 1-butanol, TBA, pentanol, phenol and 2,4-dichlorophenol; describe site specific conditions which enhance or inhibit degradation and compare biodegradation rates with thermodynamic predictions. Laboratory microcosms utilizing soil from two previously uncontaminated sites of widely varying conditions were constructed to simulate the subsurface environment. Nitrate was added to some

microcosms to stimulate denitrification and metabolic inhibitors were added to others to define conditions at each site which favor biodegradation.

Each of the test compounds except TBA was readily degraded in the Blacksburg soil. Inhibition of sulfate reduction by the addition of molybdate stimulated degradation of all compounds including TBA, whereas, inhibition of methanogenesis with BESA slowed the degradation rates. The addition of nitrate did not affect the biodegradation in Blacksburg soil. In the Newport News soil, all of the test compounds were biodegraded at substantially higher rates than was observed in the Blacksburg soil. The presence of the metabolic inhibitors did not affect degradation, however, the addition of nitrate increased the degradation rates of the alcohols but not the phenols. The degradation rates in each of the soils did not correlate with the bacterial population size or free energies of the reactions.

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Table of Contents

List of Illustrations

List of Tables

Chapter 1

Introduction

The contamination of groundwater has received widespread attention in recent years because of concern about the deterioration in the quality of drinking water supplies. Since groundwater comprises more than 95% of all available freshwater in the United States, subsurface contamination poses significant health problems. Once in the groundwater, organic compounds may migrate with the flow of water or adsorb to the soil particles. Remediation of contaminated aquifers usually requires costly treatment techniques such as activated carbon or aeration. In some cases, the aquifer must be abandoned in favor of alternate water supplies.

In recent years, researchers have searched for or attempted to culture bacteria capable of degrading man-made organic compounds as an inexpensive alternative to chemical and physical treatment of contaminated groundwater. While this type of research may eventually yield valuable results, it fails to address the fundamental question of why natural soil bacteria do not accomplish this task. The most widely held view is that soil bacteria are not acclimated to the organic compounds introduced into the environment and have not developed the enzymes necessary to degrade these chemicals (Alexander, 1981). One would expect, however, that this acclimation period would not persist indefinitely and that a population of bacteria would develop in the subsurface which

would eventually degrade xenobiotic compounds. This does not always appear to be the case. In reality, some compounds will degrade quite easily in the subsurface while others tend to persist almost indefinitely. The key questions, therefore, are why do these compounds persist and can the subsurface environment be manipulated to encourage or accelerate subsurface degradation? These questions cannot be answered with the information currently available.

This study was designed to describe the kinetics of tertiary butyl alcohol (TBA) degradation in two previously uncontaminated soils, establish the biodegradation rates of the C1 through C5 straight-chained alcohols, TBA, phenol and 2,4-dichlorophenol (DCP), describe specific conditions which favor biodegradation and compare rates with thermodynamic predictions. Methanol, ethanol and TBA are of interest because they are used as additives in some gasolines as octane boosters. In addition, methanol is used in various solvents, as a dehydrator for natural gas and in plastics. Ethanol is used in ethylene dibromide, pharmaceuticals, plastics and plasticizers, lacquers, rubber, aerosols, solvents, dyes and explosives. Propanol is used in printing inks, wool dyes, PVC adhesives, metal degreasers and in brake fluid manufacturing. 1-Pentanol (hereafter referred to as just pentanol) is used in solvents, the manufacturing of petroleum additives and as raw material for pharmaceutical preparations (Verschuerenm, 1977). 1-Butanol was included in this study for comparison with the tertiary form of butanol. These alcohols are highly soluble and do not adsorb well to soil as do more hydrophobic organics, therefore, they have the potential to migrate from any source of contamination should they be introduced into an aquifer.

Phenol is a common industrial chemical used in resins, pharmaceuticals, fungicides, dyes, herbicides and germicides. In pure cell culture studies, 64 mg/l phenol was found to inhibit cell multiplication of *Pseudomonas putida* while 10 mg/l and 3 mg/l produced similar results in the protozoa *Paramecium caudatum* and *Vorticella campanula*, respectively. DCP can be produced inadvertently by the chlorination of water containing phenol. Concentrations of 6 mg/l inhibited cell multiplication of *Pseudomonas putida*, while 2 mg/l induced a similar response in *Microcystis aeruginosa* (Verschuerenm, 1977).

By using the homologous alcohol series, the tertiary structured alcohol and the ringed compounds, it was the goal of this study to present a general picture of the mechanisms associated with subsurface degradation. In addition, factors which are and are not important in controlling degradation were to be identified.

Chapter 2

Literature Review

2.1. Introduction

Groundwater contamination by man-made organic chemicals is recognized as a serious public health threat. Groundwater accounts for 95 percent of the fresh water available in the United States and is used by about 40 to 50 percent of the people for drinking. However, between 90 and 95 percent of the people in rural parts of of the country drink groundwater with little or no treatment (Dyksen and Hess, 1982). Groundwater was once thought to be pristine. This was found not to be the case when analytical capabilities were developed which allowed for the detection of trace quantities of contaminants. Synthetic organic chemicals have been detected in many groundwater supplies as a result. In 45% of the large public water supplies which utilize groundwater, volatile organic chemicals have been detected (Wilson, et al., 1983b). The problem lies in the fact that the subsurface does not have an effective self cleansing mechanism, therefore, groundwater may remain contaminated for many years. Magnifying the problem is our general lack of knowledge about the subsurface. Only within the last decade have methods been developed for analyzing the subsurface

and determining the factors which control the transport and fate of anthropogenic chemicals in groundwater.

Most of the knowledge gathered thus far on biodegradation has been developed primarily using pure cultures of organisms degrading individual organic compounds or batch and column studies using seeded bacterial populations or enrichment cultures. The results from these studies may not translate well to the complex subsurface environment. Some studies have been performed using aquifer material and the indigenous bacterial population to study biodegradation. In each case, however, the outcome of the study was to determine whether compounds were degradable but little rate data has been generated. It was determined that biodegradation was site specific in most instances. Since direct investigation of each man-made organic chemical at every site of interest would be prohibitively expensive, new approaches to the questions concerning biodegradation must be developed. One aspect of subsurface biodegradation which has received little attention is the role of the aquifer chemistry. The chemistry of an aquifer and the influence chemistry has on the competing metabolic processes may be important in determining why some anthropogenic compounds persist. This type of information may allow for defining methods for improving degradation, predicting the rates of degradation and evaluating hazardous waste sites in terms of their degradation potential.

2.2. Methods to Study the Subsurface

The study of subsurface processes began with the development of techniques for recovering soil which avoided contamination by the sampling process. Procedures developed by Dunlap, et al. (1977) and modified by Wilson, et al. (1983b), Bengtsson (1985) and Novak, et al. (1985) involved boring to a desired depth with an auger and collecting the sample in a sterile, thin-walled, core barrel. The core material from the tube was extruded and pared to remove any material which would be contaminated by the sampling operation. Ghiorse and Balkwill (1981 and 1983) deter-

mined that these samples could be kept at 4 °C for 6 months or more without altering the bacterial population.

The primary method for studying degradation in the subsurface system is with microcosms. Bengtsson (1985) defined the microcosm as a part of the ecosystem which can be controlled in the laboratory due to its reduction in size and complexity. It can be used to isolate various physical parameters and estimate the response of the larger system. This capability allows the researcher to collect a significant amount of information inexpensively. Ausmus, et al. (1980), however, has identified problems which should be taken into account when evaluating data obtained from microcosms. The first is the uncertainty of applying data obtained from the simplified system to the more complex natural system. The second problem is a lack of consistency in the size and structural characteristics of microcosms. This may affect the ability to reproduce and compare results. The third problem is in establishing confidence limits between data obtained from replicate microcosms.

Wilson and Noonan (1984) have described two broad classes of microcosms. The first focuses on the biological response to changes in the environment. The second emphasizes the pollutant and its interactions with the environment. This second type of microcosm can be designed either to imitate the response of the larger system or to obtain values for mathematical expressions which will be used to predict the response of the larger system. The design of a microcosm, therefore, depends on the type of information desired. Wilson, Noonan and McNabb (1981) and Novak, et al. (1985) described simple, inexpensive microcosms for monitoring the biodegradation of organic compounds by soil bacteria. These systems utilized screw-capped test tubes containing aquifer material mixed with the test compounds of interest. The disadvantage of this type of microcosm is that it does not simulate the flow of groundwater. It is, however, a quick and relatively inexpensive way of analyzing organic compounds and subsurface materials while easily maintaining anoxic conditions.

More complicated microcosms which simulate the flow of water have been developed by Dunlap, et al. (1972) and Bengtsson (1981). These designs, however, are difficult to build and operate. The materials are expensive and the maintenance time is considerable. Anaerobic conditions are also difficult to maintain. The potential for contamination by outside organisms is increased because of the continual need to prepare feed solution. These systems, however, are well suited when precise results are desired and batch microcosms are not suitable.

2.3. Microbial Activity in the Subsurface

Researchers have been able to characterize indigenous soil bacteria as result of the development of techniques for aseptically sampling the subsurface. Using electron microscopy, Ghiorse and Balkwill (1981, 1983 and 1985), Balkwill and Ghiorse (1985) and Wilson, et al. (1983a) observed that a sandy aquifer contained a morphologically diverse bacterial population with very few eukaryotic organisms. The bacteria found were generally smaller than laboratory cultivated cells and contained both gram-positive and gram-negative types. Gram-positive bacteria were more abundant. Cell numbers were determined by the acridine-orange (AO) fluorescent direct counting technique and the number of viable organisms were determined by plate-count techniques using two types of media. In each case, the number of colony forming units (cfu) from the plate-counts were less than the AO-fluorescent direct counts. This either indicated that many of the bacteria were not viable or that the media used was selective for certain types of bacteria.

At two Oklahoma sites and one in Louisiana, the number of cells determined by the AO epifluorescent direct count ranged from 1 to 10 million/gram of dry soil while the number of viable organisms determined by plate counts varied significantly depending on the site and the media used. The number of viable bacteria expressed as cfu/gram of dry soil ranged from 100 to 1000 times less than the number of cells determined by the AO epifluorescent direct count at the Oklahoma site. At the Louisiana site, the number of viable bacteria were about 10,000 times less than the total number. These results were determined using a media containing peptone, yeast extract and

glucose. In each case, a dilute media yielded higher counts than a concentrated media. The authors attributed this result to the oligotrophic nature of subsurface bacteria.

Novak, et al. (1985) and Goldsmith (1985) determined that in soil from Pennsylvania, New York and Virginia, the number of cells as determined by the AO epifluorescent technique did not vary significantly between sites or with depth. Viable cell counts using a soil extract media, however, varied by as much as three orders of magnitude throughout the soil profile. The plate count values were one to four orders of magnitude less than the number of cells determined by direct counts. Goldsmith reported bacterial numbers as a function of depth for subsurface material collected at the three sites. In the Williamsport, Pennsylvania soil, $5.6 \pm 1.9 \times 10^7$ cfu/g soil were detected at the surface, while $3.9 \pm 1.4 \times 10^7$ and $4.6 \pm 2.7 \times 10^7$ cfu/g soil were measured at 12 and 30 feet, respectively using the A-O technique. Using plate counts with soil extract media, $3.0 \pm 0.3 \times 10^7$ cfu/g soil were measured at the surface, $3.5 \pm 2.1 \times 10^3$ cfu/g soil at 12 feet and $1.4 \pm 0.8 \times 10^5$ cfu/g soil at 30 feet. For the Wayland, New York soil, the A-O technique resulted in $1.0 \pm 0.4 \times 10^8$ cfu/g soil at the surface, $7.6 \pm 3.8 \times 10^7$ cfu/g soil at 6 feet and $8.0 \pm 6.4 \times 10^7$ cfu/g soil at 12 feet. Soil extract plate counts at the same depths yielded $1.0 \pm 0.4 \times 10^6$ cfu/g soil, $9.3 \pm 1.1 \times 10^5$ cfu/g soil and $1.1 \pm 0.1 \times 10^5$ cfu/g soil. At the Dumfries, Virginia site, $1.0 \pm 0.4 \times 10^8$ cfu/g soil were measured at the surface (A-O technique), while between $3.1 \pm 2.2 \times 10^7$ cfu/g soil and $1.1 \pm 0.6 \times 10^8$ cfu/g soil were detected from 11 to 102 feet. Plate counts with soil extract yielded counts averaging two orders of magnitude less than the direct counts. The only exception was at 11 feet where less than 10^3 cfu/g soil were detected with plate counts. Goldsmith reported that the acridine-orange counts were more indicative of the actual bacteria population because of the biases introduced by plate counting media.

White (1986) measured the bacterial population in a gasoline contaminated soil from Philadelphia, Pennsylvania. At the surface, 6.8×10^7 organisms per gram soil were detected using the A-O direct count while plate counts yielded 4.3×10^7 and 3.9×10^7 cfu per gram soil using soil extract and yeast extract media, respectively. The counts did not vary by more than one order of magnitude throughout the soil profile. White indicated that direct counts yielded misleading results

in clay soil because of difficulty distinguishing between bacteria and clay particles. For non-clay soils, there was little difference between the A-O and plate count results. Overall, however, plate counts were considered more reliable.

Hirsh and Rades-Rohkohl (1983) observed 90 morphologically diverse organisms in groundwater obtained from a 10 meter well in a study of microbial diversity of the subsurface. Of these, 72 organisms were bacteria, 10 were protozoa and 8 were fungi. Most of the bacteria were covered with a surface polymer which may indicate a high degree of surface attachment and aggregation. In these experiments, 10 types of media were used. The highest number of different organisms were obtained with a media containing peptone, yeast extract, glucose and Hutners basal salt solution. Most bacteria grew well on dilute media which, like Ghiorse and Balkwill, the authors attributed to the oligotrophic nature of subsurface organisms.

Oligotrophic bacteria are characterized by their ability to grow in nutrient limiting conditions. Many of them, however, do not grow in high concentrations of organic substrates. Oligotrophic bacteria gain their competitive advantage through their ability to rapidly reproduce at low organic concentrations. The inability of oligotrophic bacteria to grow in high nutrient environments may be due to an inefficient process for disposing of hydrogen peroxide generated during metabolism. In experiments using *Leptothrix pseudoochraceae, Siderocapsa eusphaera* and *Metallogenium personatum*, high concentrations of nutrients resulted in an accumulation of hydrogen peroxide in the medium and lysed cells. These organisms grew in the presence of an abundance of nutrients if catalase was added. If a culture of organisms with a high extracellular catalase activity was added, the oligotrophic bacteria grew in a high organic medium (Kuznetsov, Dubinina and Lapleva, 1979).

2.4. Denitrification

The reduction of nitrate serves two functions in bacterial metabolism, assimilatory reduction to ammonia and eventually to the amino acid and protein level; and dissimilatory reduction in which

oxidized nitrogen serves as an electron acceptor during the mineralization of organic compounds. It is the dissimilatory process which is of interest in biological degradation. Most all bacteria can reduce nitrate for assimilating purposes, but not all species are able to utilize nitrate as an electron acceptor. The majority of these are facultative, using oxygen as an electron acceptor in an aerobic environment, then switching to nitrate when oxygen becomes limiting (Delwiche and Bryan, 1976). Denitrifying bacteria include species of *Paracoccus, Thiobacillus, Pseudomonas, Hypomicrobium, Alcaligenes* and *Bacillus*. Other species can reduce nitrate but not nitrite. These include *Escherichia, Spinillum, Propionobacterium, Corynebacterium* and *Streptococcus* (Whatley, 1981).

The process of denitrification as a repository of electrons during organic matter oxidation is demonstrated below:

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G^\circ = -17.1 \text{ kcal} \qquad (2.4a)$$

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{4}H_2O \rightarrow \frac{1}{4}CO_2 + H^+ + e^- \qquad \Delta G^\circ = -10.0 \text{ kcal} \qquad (2.4b)$$

Combining equation 2.4a and 2.4b yields:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{5}NO_3^- + H^+ \rightarrow \frac{1}{4}CO_2 + \frac{1}{10}N_2 + \frac{3}{5}H_2O \quad \Delta G^\circ = -27.1 \text{ kcal} \qquad (2.4c)$$

The negative free energy value for equation 2.4c indicates that the reaction is favored in the direction written.

The denitrification pathway has not been clearly defined. The differences in pathways, however, may reflect differences among bacterial species rather than inconclusive data. It is generally accepted that reduction of nitrate to nitrite is the initial step in pathway. The enzyme which catalyzes this reaction, nitrate reductase, is formed only under conditions in which dissimilatory nitrate reduction can take place. In *Bacillus stearothermophilus*, the presence of nitrate induces enzyme production (Downey, Kiszkiss and Nuner, 1969). In *Bacillus licheniformis*, however, the enzymes

are produced in anaerobic conditions regardless of whether nitrate is present (Payne, 1973). This enzyme contains molybdenum (Delwiche and Bryan, 1976).

The reduction of nitrite to N_2 is not completely understood. Some researchers speculate that hyponitrous acid and nitroxyl are likely intermediates, however, these compounds are unstable making their detection difficult. Nitric oxide has also received consideration as a possible intermediate. In enzyme extracts, NO has been detected as the reduction product of NO_2^- (Renner and Becker, 1970; Payne, Riley and Cox, 1971), however, there is no evidence in cell cultures that NO accumulates during denitrification (Whatley, 1981). Nitrous oxide appears to be a common, though not universal, intermediate in the reduction of nitrite to N_2 (Delwhiche and Bryan, 1976).

Denitrifying bacteria are common to most soils. Bollag, Orcutt and Bollag (1970) isolated 60 different nitrate reducing organisms in soil. Volz, et al. (1975) estimated the maximum number of nitrate reducing bacteria to be 6.9×10^5/g soil in a ponded sandy loam. Of these, 2.9×10^4/g soil were denitrifying organisms. The number of nitrate reducing and denitrifying bacteria decreased with depth up to 100 cm. Knowles (1981) determined that the rate of denitrification decreased with increasing amounts of oxygen. Loss of nitrogen, however, was detected during aeration presumably due to the presence of anaerobic microsites. The existence of these sites prevent accurate measurement of the optimal E_h for denitrification, however, average values in the bulk soil indicate that active denitrification occurs at a redox potential of + 200 mV with the process complete when the E_h reaches + 100 mV (Knowles, 1981).

Environmental factors appear to affect nitrite reduction more than nitrate reduction. Bollag, et al. (1970) reported that at pH 5 there was little reduction activity in four cultures isolated from soil. At pH 6, nitrate was reduced in 2 of the 4 cultures, however, nitrite accumulated in the system. Optimum growth for all 4 organisms occurred at pH 7. Knowles (1981), on the other hand, reported the buildup of N_2O in nitrate reducing systems at pH 4, but acknowledged the possibility of NO_2^- accumulation in some systems. N_2O also accumulates in systems with high nitrate content such as fertilized soils. This phenomenon is accentuated in low pH conditions (Blackmer and

Bremner, 1978). In a study of denitrification activity as indicated by denitrifying enzyme quantity, Tiedje, et al. (1982) measured a significant denitrification capacity in agricultural soils (pH 5.8-6.6) swamp sediment (pH 7.7) and forest soils (pH 4.0-6.4). Reduction in the low pH forest soil was a surprising result. They also observed a strong correlation between organic carbon and denitrification capacity. The authors speculated that organic carbon may exert a greater influence on denitrification than does oxygen.

Denitrification has been observed to influence other reduction processes. In many studies, the presence of nitrate was inhibitory to methane production and sulfate reduction (Barker, 1941; Takai, Koyama and Kamura, 1956; Yamane, 1957; Laskowski and Monaghan, 1967; Bell, 1969; Cappenburg and Patrick, 1969; Macgregor and Keeney, 1973; and Bollag and Czlonkowski, 1973). Nitrate reduction maintains the E_h at a level inhibitory to methanogenic and sulfate reducing bacteria. Jenneman, McInerney and Knapp (1986) proposed that the presence of N_2O may exert an inhibitory effect on sulfate reduction in addition to increasing the E_h.

2.5. Sulfate Reduction

Sulfate is reduced by two different methods. Assimilatory sulfate reduction involves the conversion of inorganic sulfate to organic sulfur for use by the organism in its metabolic process. In this process, the organism converts sulfate to hydrogen sulfide intracellularly for incorporation as sulfhydryl groups in the amino acids cysteine and methionine.

On the other hand, sulfate is used as an electron acceptor during the oxidation of organic matter in dissimilatory sulfate reduction. Hydrogen sulfide is produced as an end product and released into the surrounding environment. By examining the thermodynamics of dissimilatory reduction, the use of sulfate as an electron acceptor can be demonstrated:

$$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- \rightarrow \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{2}H_2O \qquad \Delta G^\circ = 5.1 \text{ kcal} \qquad (2.5a)$$

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{4}H_2O \rightarrow \frac{1}{4}CO_2 + H^+ + e^- \qquad \Delta G^\circ = -10.0 \text{ kcal} \qquad (2.5b)$$

Combining equations 2.5a and 2.5b yields:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ \rightarrow \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{4}CO_2 + \frac{1}{4}H_2O \qquad (2.5c)$$

$$\Delta G^\circ = -4.9 \text{ kcal}$$

The negative free energy value for reaction 2.5c indicates that this reaction is favored in the direction written. Whether the reaction actually proceeds depends on the presence of biological enzymes which mediate the reaction, proper environmental conditions and a supply of organic substrates.

Bacteria of the genus *Desulfovibrio* are the predominant sulfate reducing bacteria with the specie *desulfuricans* the most common. Other species include *Desulfotomaculum, Desulfonema, Desulfococcus* and *Desulfosarcina* (Thauer and Badziong, 1981). All are obligate anaerobes. Sulfate reduction, in general, is enhanced in warm, wet or water logged conditions (Alexander, 1977). In a study using waterlogged soil, Connell and Patrick (1968) determined that no sulfide would accumulate until the redox potential was less than -150mV. As the redox potential was decreased between -150mV and -300mV, the level of sulfide increased. They also determined that no sulfide was produced if the pH was less than 6.5 or greater than 8.5. In another study by Connell and Patrick (1969), the addition of reducible iron resulted in a decrease in the H_2S concentration presumably by precipitation of iron sulfide. Furthermore, when H_2S was added to the system, the amount of H_2S removed by precipitation was equal to the ferrous iron released from the soil.

There is no absolute agreement on the mechanism of dissimilatory sulfate reduction. The three proposed pathways are as follows:

$$SO_4^{-2} \xrightarrow{ATP} SO_3^{-2} \rightarrow S_3O_6^{-2} \rightarrow S_2O_3^{-2} \rightarrow S^{-2} \qquad (2.5d)$$

$$SO_4^{-2} \xrightarrow{ATP} SO_3^{-2} \rightarrow S_2O_3^{-2} \rightarrow S^{-2} \qquad (2.5e)$$

$$SO_4^{-2} \xrightarrow{ATP} SO_3^{-2} \rightarrow S^{-2} \qquad (2.5f)$$

The first pathway was originally proposed by Kobayashi, Tachibana and Ishimoto (1969) utilizing pure cultures of *Desulfovibrio vulgaris*. In experiments using enzyme extracts from *Desulftomaculum nigrificans*, Akagi, Chan and Adams (1974) detected thiosulfate as an intermediary in the reduction of sulfate but did not observe trithionate. Their conclusions are expressed in the second pathway given above. In a subsequent study, Kobayashi and coworkers (1974) reevaluated their previous findings and reported that thiosulfate and trithionate are not intermediates in dissimilatory sulfate reduction. This was also reported by Chambers and Trudinger (1975). These conclusions are reflected in the third pathway given above.

Researchers originally believed that carbon metabolism by sulfate reducing bacteria was incomplete, yielding short chain fatty acids, alcohols and sugars (Postgate, 1965). In recent years, some sulfate reducers have been found to oxidize short chain fatty acids, in particular acetate. Widdel and Pfennig (1977) first isolated an acetate oxidizing sulfate reducer, *Desulfomaculum acetoxidans*. The list of bacteria with this ability was extended to include *Desulfonema limicola, Desulfovibrio baarsii, Desulfococcus multivorans, Desulfosarcina variablis* and *Desulfonema magnum* (Pfennig and Widdel, 1981; and Laanbroek and Pfennig, 1981). This reaction is given as follows:

$$CH_3COO^- + SO_4^{-2} \rightarrow S^{-2} + CO_2 + HCO_3^- + H_2O \quad \Delta G^\circ = -14.47 \text{ kcal} \qquad (2.5g)$$

These studies demonstrated conclusively that sulfate reduction could result in the terminal oxidation of organic matter. Table 1 gives a list of sulfate reducing bacteria and substrates used for complete mineralization of carbon compounds.

Sulfate reducers have been found to use hydrogen as an electron donor during the incomplete oxidation of simple alcohols and fatty acids (Postgate, 1979). In addition, Badziong, Thauer and

Table 1. Species of sulfate reducing bacteria which degrade fermentation products.

Species	Substrate
Desulfobacter postgatei	Acetate
Desulfotomaculum acetoxidans	Acetate
Desulfococcus multivorans	Formate Acetate Propionate
Desulfosarcins variabilis	Hydrogen Formate Acetate Propionate
Desulfonema magnum	Hydrogen Formate Acetate Propionate
Desulfovibrio baarsii	Formate Acetate Propionate
Desulfobulbus propionucus	Propionate

Zeikus (1978) determined that *Desulfovibrio vulgaris* isolated from sewage digester sludge and a eutrophic pond grew with hydrogen and sulfate as the sole energy source. This reaction is given as:

$$4H_2 + SO_4^{-2} \rightarrow H_2S + 2H_2O + 2OH^- \qquad \Delta G^\circ = -36.4 \text{ kcal} \tag{2.5h}$$

Acetate and carbon dioxide served as carbon sources. Brandis and Thauer (1981) confirmed these results using *Desulfovibrio vulgaris, D. desulfuricans* and *D. gigas*. These studies demonstrated that sulfate reduction was an energy conserving process similar to nitrate reduction and oxygen respiration (Pfennig and Widdel, 1982).

Group VI anions such as chromate, tungstate and especially molybdate, are used to inhibit sulfate reduction in many studies. This inhibition results from the stereochemical similarity between the group VI anions and the sulfate ion. The specific effect is related to the first step of the reduction pathway. In this reaction, sulfate is activated to adenosine 5'-phosphosulfate (APS) by adenosine 5'-triphosphate (ATP) sulfurylase. This reaction is given below:

$$SO_4^{-2} + ATP \rightarrow APS + PP \tag{2.5i}$$

Group VI anions are also substrates for this reaction converting ATP to AMP and pyrophosphate (PP) destroying ATP and preventing sulfate uptake and utilization. This was confirmed in a study of ATP levels in the sulfate reducing bacteria *Desulfovibrio* with and without molybdate. The ATP concentration as measured by the firefly assay decreased by 90% in the presence of molybdate. ATP levels in aerobes, nitrate reducers and fermenters were not affected or slightly increased in the same conditions (Taylor and Oremland, 1979). Banat, et al. (1981 and 1984) determined that molybdate inhibited acetate degradation in salt marsh sediment. Without molybdate, acetate was degraded and hydrogen sulfide was produced. Molybdate had no effect in sediment with no sulfate. Smith and Klug (1981) reported that 0.2 mM molybdate inhibited sulfate reduction in eutrophic lake sediments, however, methanogenesis was relatively unaffected. Similar results were also reported by Winfrey and Ward (1983) and Alperin and Reeburgh (1985).

An important aspect of dissimilatory sulfate reduction is the control it can exert on the carbon and electron flow in aquatic systems. Sulfate reduction has been shown to inhibit other forms of metabolism, in particular methanogenesis (Winfrey and Zeikus, 1977; Abram and Nedwell, 1978; Mountfort, Asher, Mays and Tiedje, 1980; Smith and Klug, 1981; Sorensen, Christensen and Jorgensen, 1981; Oremland and Polcin, 1982; Lovley and Klug, 1983; and Winfrey and Ward, 1983). In each of these studies, the sulfate reducing bacteria successfully competed for the available H_2 and acetate with a resulting suppression of the methane producing bacteria. Winfrey and Zeikus (1977) determined that methanogenesis involving H_2 and acetate was inhibited by as little as 0.2 mM sulfate. The flow of carbon and hydrogen was shifted to methanogenesis with the subsequent production of methane once the sultate had been consumed. Oremland and Polcin (1982), on the other hand, reported that the methanogenesis of methanol and methionine was not inhibited by the presence of sulfate ions. They described substrates such as acetate and H_2 for which methanogenesis was inhibited by sulfate reduction as competitive; whereas, substrates such as methanol and methionine were described as noncompetitive.

Several theories have been proposed to explain the apparent inhibition of methanogenesis by sulfate reduction. Cappenberg (1974) proposed that the presence of sulfide was toxic to methane producing bacteria. MacGregor and Keeney (1973) contended that sulfate reduction increased the sediment E_h to a level inhibitory to methanogenesis. Winfrey and Zeikus (1977) disputed each of these theories. They contended that the small concentration of sulfate necessary to inhibit methanogenesis would not significantly alter the E_h. They also were unable to detect a significant amount of sulfide due to precipitation of metal sulfides and, therefore, concluded that there was not enough free sulfide in their system to affect the methanogenic bacteria. Though Winfrey and Zeikus do not propose a mechanism of their own, they do point out that sulfate inhibition of methanogenesis is compatible with the thermodynamics of the system. Their calculations are given as follows:

$$SO_4^{-2} + CH_3COO^- + H^+ \rightarrow H_2S + 2HCO_3^- \qquad \Delta G = 47.3 \text{ J} \qquad (2.5j)$$

$$CH_3COOH \rightarrow CO_2 + CH_4 \qquad \Delta G = 28.5 \text{ J} \qquad (2.5k)$$

Sulfate reduction of acetate as illustrated by equation 2.5j above yields a greater free energy than does the methanogenic reaction shown in equation 2.5k. Likewise, the reduction of sulfate by H_2 yields 154.0 J, whereas, the reduction of CO_2 by H_2 to methane yields 135.1 J.

Kristjansson, Schonheit and Thauer (1982) and Schonheit, Kristjansson and Thauer (1982) attributed the competitive advantage of sulfate reducers over methane producers for H_2 and acetate to the difference in substrate affinities as expressed by the value of the half-saturation constant (K_s). Using pure cultures of *Desulfovibrio vulgaris* and *Methanobrevibacter arboriphilus*, the rate of H_2 usage was five times greater for the sulfate reducing bacteria than for the methane producer when the concentration of H_2 was limiting. The K_s was 1μm for *D. vulgaris* and 6μm for *M. arboriphilus*. Similarly, when acetate was used in limiting quantities, the K_s for the sulfate reducer was 0.2 mM and 3 mM for the methane producer.

2.6. Methanogenesis

Methanogenesis involves a bacterial consortium of methanogenic and chemoheterotrophic, nonmethanogenic bacteria. Methane producing bacteria require an anoxic environment which is without light and alternate electron acceptors to CO_2 such as nitrate and sulfate. The redox potential must be less than about -200mV (Bell, 1969, Connell and Patrick, 1969, and Cappenburg, 1974). In the first step, chemoheterotrophic, nonmethanogenic bacteria convert complex organics to volatile acids, alcohols, hydrogen and carbon dioxide. The alcohols and volatile fatty acids which are longer than two carbons are converted to hydrogen and acetic acid by a class of bacteria called acetogens. Methanogenic bacteria utilize the acetic acid and hydrogen to produce methane (Speece, 1983).

Initial studies into the process of methanogenesis began by determining the source of methane. Barker (1936) proposed that all biologically produced methane originated from the reduction of carbon dioxide. Buswall (1939), on the other hand, contended that methane was derived from the methyl group of acetic acid, while CO_2 came from the carboxyl group. It was not until 1948 that Buswall and Sallo used C^{14} tracer experiments to confirm Buswall's earlier contention that biologically produced methane did indeed originate from the methyl group of acetate. This finding was also confirmed by Stadtman and Barker (1949). In additional studies, Stadtman and Barker (1951) determined that methane was produced from CO_2 reduction when ethanol, n-butanol, propionate, butyrate and caproate were used as the initial substrates. Methanol was also identified as a source of methane. The following methanogenic reactions, therefore, were proposed from these early studies.

$$C^{14}H_3COOH \rightarrow C^{14}H_4 + CO_2 \qquad (2.6a)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad (2.6b)$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad (2.6c)$$

Jeris and McCarty (1965) determined that the conversion of certain complex organics to methane involved a common intermediate, acetate. Using radiolabeled carbon dioxide with ethanol as the only substrate, the following equations were developed:

$$2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COOH + 8H \qquad (2.6d)$$

$$8H + C^{14}O_2 \rightarrow C^{14}H_4 + 2H_2O \qquad (2.6e)$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \qquad (2.6f)$$

Combining equations 2.6d, 2.6e and 2.6f yields:

$$2CH_3CH_2OH \rightarrow C^{14}H_4 + 2CH_4 + CO_2 \qquad (2.6g)$$

These results explained and confirmed the findings of Stadtman and Barker (1949).

Jervis and McCarty determined that long chain fatty acids were degraded by β-oxidation in which the beta carbon to the carboxyl group of the fatty acid is oxidized. When an even numbered carbon was radiolabeled, the result was radioactive methane, however, if an odd numbered carbon was labeled, radioactive CO_2 was detected. This fact is illustrated in the following equations using octanoic acid as the substrate.

$$CH_3C^xH_2(CH_2)_4C^oH_2COOH + 6H_2O \rightarrow CH_3C^xOOH + C^oH_2COOH + 2CH_3COOH \quad (2.6h)$$

$$CH_3C^xOOH \rightarrow CH_4 + C^xO_2 \quad (2.6i)$$

$$C^oH_3COOH \rightarrow C^oH_4 + CO_2 \quad (2.6j)$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (2.6k)$$

Similar results were obtained for glucose establishing acetate as a primary intermediate in methanogenesis.

According to McCarty and Smith (1986), the production of methane from a mixture of complex organics such as the anaerobic digestion of sewage sludge involves propionate as an intermediate. As illustrated in Figure 1, approximately 30% of the electrons associated with methane formation flows through propionate. Under standard conditions, this reaction is thermodynamically unfavorable. As the concentration of H_2 is reduced, however, the reaction proceeds. For propionate, as the H_2 partial pressure is reduced between about 10^{-4} atm. and 10^{-6} atm., the ΔG for the reaction becomes increasingly negative.

In early methanogenic studies, Barker (1940) used what was thought to be a pure culture of *Methanobacterium omelianskii.* Bryant, et al. (1967), however, determined that this organism was

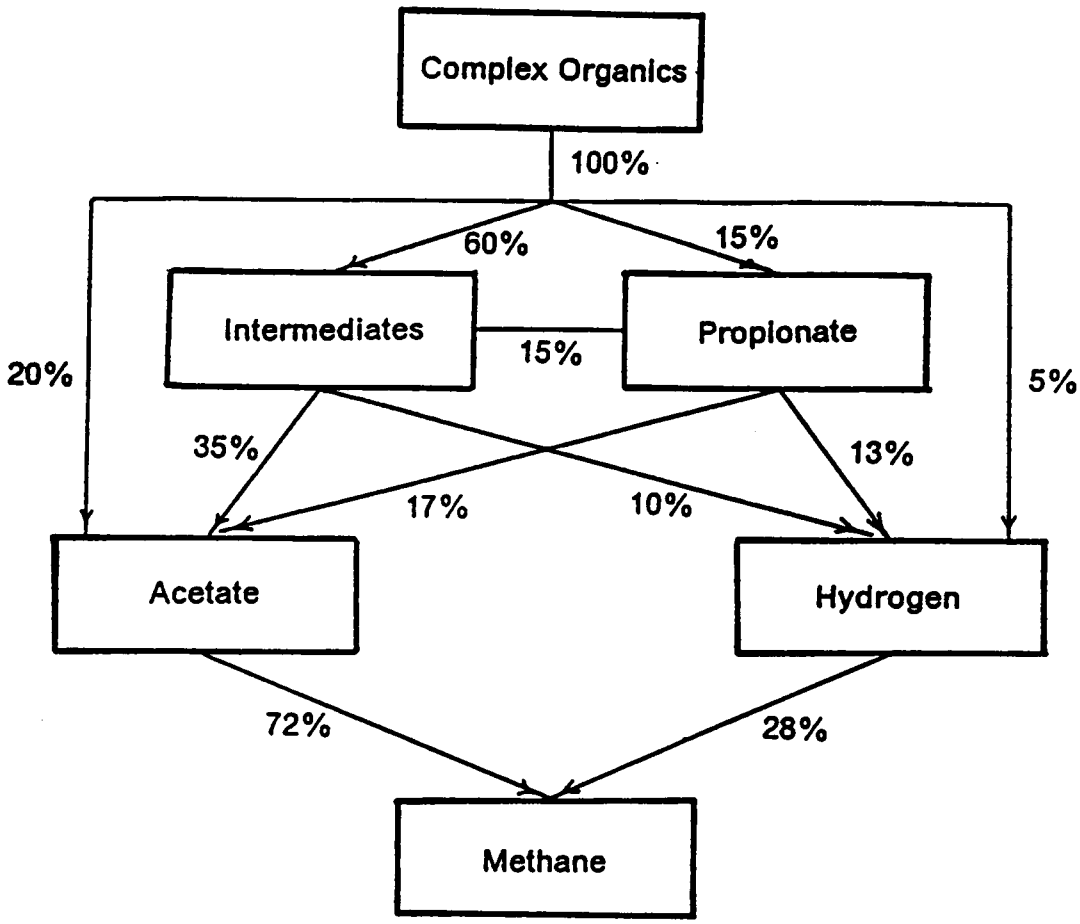

Figure 1. Electron flow during methanogenesis (after McCarty and Smith, 1986).

in fact a mixed culture of two species existing in a symbiotic association. The first species designated S organism produced H_2 according to the following reaction:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2 \quad \Delta G^\circ = +1.5 \text{ kcal} \tag{2.6l}$$

The formation of H_2 in this reaction is essential to dispose of electrons generated in the oxidation of the initial substrate to acetate. This transfer of electrons through the reduction of protons is accomplished through a nicotinamide dinucleotide dependent reaction (Reddy, et al, 1972). These reactions are given as follows:

$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+ \quad \Delta G^\circ = +5.7 \text{ kcal} \tag{2.6m}$$

$$NADH + 2H^+ \rightarrow NAD^+ + H_2 \quad \Delta G^\circ = +4.3 \text{ kcal} \tag{2.6n}$$

Under standard conditions, this reaction is thermodynamically unfavorable as indicated by the positive value of the free energy. As the H_2 partial pressure is reduced, however, the reaction becomes more favorable as the free energy change becomes increasingly negative. S organism, therefore, grows poorly unless an H_2 utilizing organism is present. In the *Methanobacillus omelianskii* culture, the H_2 utilizing organism has been designated *Methanobacterium* strain MOH. This species mediates the following reaction:

$$CO_2 + 4H_2 \rightarrow CH_4 + H_2O \quad \Delta G^\circ = -33.2 \text{ kcal} \tag{2.6o}$$

The H_2 removed in this reaction creates thermodynamically favorable conditions for S organism which in turn provides more H_2 for strain MOH. This phenomena has been termed interspecies hydrogen transfer. According to Mah, et al. (1977), interspecies H_2 transfer provides a mechanism for:

1. utilizing low molecular weight, unfermentable organics as carbon and energy sources.
2. increasing cell yield of the H_2 producer by increasing the energy yield per mole of substrate fermented.

3. providing methanogenic bacteria with usable substrates (H_2 and CO_2) from complex organic compounds such as carbohydrates, fatty acids and proteins.

Methanogenic bacteria, therefore, can only use a few substrates and depend on its symbiotic relationship to provide these from more complex organics. Table 2 lists the substrates used by methanogenic bacteria.

The conversion of acetate to methane and carbon dioxide as indicated in equation 2.6a involves oxidation of the carboxyl group to CO_2 followed by reduction of the methyl group (Mah, et al. 1977, Mah, et al. 1978, and Smith and Mah, 1978). The oxidation of the carboxyl group provides a source of electrons for the reduction of the methyl group. The formation of methane requires the presence of a methyl carrier compound called 2-mercaptoethanesulfonic acid or coenzyme M ($HSCH_2CH_2SO_3^-$ abbreviated HS-CoM). This compound is sensitive to oxygen but resistent to heat or acid. In C^{14} tracer studies, 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM) has been identified as the first product of CO_2 reduction in cell extracts. The proposed methyl reduction reaction, therefore, has been described as follows:

$$CH_3OH + HS - CoM \rightarrow CH_3 - S - CoM + H_2O \qquad (2.6p)$$

$$CH_3 - S - CoM \rightarrow CH_4 + HS - CoM \qquad (2.6q)$$

Reaction 2.6q requires a hydrogen source plus ATP and Mg^{+2}.

2-Bromoethanesulfonic acid (BESA) is used as an inhibitor of methanogenesis in many studies. Smith and Mah (1978) reported that the addition of 70 μM of BESA to *Methanosarcina* strain 227 resulted in a 75% decrease in methane production as compared with untreated controls. Addition of coenzyme M alleviated the inhibition. These results suggested that 2-bromoethanesulfonic acid is an analog of CoM (2-mercaptoethanesulfonic acid) and competes specifically in the methyl carrier step of methanogenesis. Zehnder and Brock (1979) reported 100% inhibition of methane formation from acetate in the presence of 1 mM BESA. Smith and Mah (1981) discovered that 1 μM

Table 2. Substrates used by methanogenic bacteria

Bacteria	Substrate	Reference
Methanosarcina strain	acetate	Smith and Mah, 1978
	methanol	Mah, et al. 1978
Methanobacterium nantium	H_2/CO_2	Zehnder and Brock, 1979
	formate	
Methanobacterium MOH	H_2/CO_2	Zehnder and Brock, 1979
Methanobacterium formicicum	formate	Zehnder and Brock, 1979
	H_2/CO_2	
Methanobacterium arbophilicum	H_2/CO_2	Zehnder and Brock, 1979
Methanobacterium AZ	H_2/CO_2	Zehnder and Brock, 1979
Methanobacterium barkeri	acetate	Zeikus, et al. 1975
	H_2/CO_2	Zehnder and Brock, 1979
	methylamine	Hippe, et al. 1977
	dimethylamine	Hippe, et al. 1977
	trimethylamine	Hippe, et al. 1977
Methanobacterium thermoautotrophicum	acetate	Zeikus, et al. 1975
	H_2/CO_2	Zehnder and Brock, 1979
	CO	Daniels, et al. 1977

BESA inhibited methanogenesis from H_2/CO_2, methanol, methylamine, trimethylamine and acetate though 10 μM was required for complete inhibition. When BESA and CoM were added in combination, there was no inhibition. In a mixed culture, methanogenic fixed-film column, Bouwer and McCarty (1983a) reported 41% inhibition using 6 x 10^{-4} M BESA.

2.7. Biological Energetics

Bacterial growth is related to the free energy released during the oxidation of organic material and the efficiency with which organisms are able to capture and utilize this energy (Stumm and Morgan, 1981). The oxidation of organic matter is coupled with the reduction and transfer of electrons to primarily oxygen, nitrate, sulfate or carbon dioxide. Oxygen as an electron acceptor yields the most free energy during oxidation of organic matter, whereas, carbon dioxide reduction yields the least. Aerobic systems, therefore, typically yield more biomass than do methanogenic environments. This is illustrated below with acetate as a model compound (McCarty, 1972).

$$\frac{1}{8}CH_3COO^- + \frac{1}{4}O_2 \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + \frac{1}{8}H_2O \qquad (2.7a)$$

$$\Delta G_r = -25.28 \text{ kcal/electron equivalent}$$

$$\frac{1}{8}CH_3COO^- + \frac{1}{2}NO_3^- \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + \frac{1}{8}H_2O + \frac{1}{2}NO_2^- \qquad (2.7b)$$

$$\Delta G_r = -16.03 \text{ kcal/electron equivalent}$$

$$\frac{1}{8}CH_3COO^- + \frac{1}{4}SO_4^{-2} + \frac{3}{16}H^+ \rightarrow \frac{1}{8}CO_2\frac{1}{8}HCO_3^- + \frac{1}{8}H_2O + \frac{1}{16}H_2S + \frac{1}{16}HS^- \qquad (2.7c)$$

$$\Delta G_r = -1.52 \text{ kcal/electron equivalent}$$

$$\frac{1}{8}CH_3COO^- + \frac{1}{8}H_2O \rightarrow \frac{1}{8}CH_4 + \frac{1}{8}HCO_3^- \qquad (2.7d)$$

$$\Delta G_r = -0.85 \text{ kcal/electron equivalent}$$

The value of the free energy for these reactions was determined assuming unit activity for each compound except hydrogen. The pH of the system was assumed to be neutral, therefore, the activity of hydrogen was 10^{-7}. Finally, an electron at unit activity has a zero free energy. Whether these reactions occur or not, however, depends on many factors besides free energy. There must be bacteria present capable of producing enzymes which catalyze the reaction. Furthermore, environmental conditions must be favorable to allow the survival of the proper bacteria.

McCarty (1969, 1972 and 1975) has developed relationships which use energy considerations to estimate bacterial growth and subsequently substrate utilization rates. The microorganism growth rate given by McCarty is:

$$\frac{dX_a}{dt} = a_e \frac{dF}{dt} - bX_a \tag{2.7e}$$

where:

$\frac{dX_a}{dt}$ = biomass growth rate, mass volume^{-1} time^{-1}

X_a = biomass concentration, mass volume^{-1}

a_e = growth yield coefficient

$\frac{dF}{dt}$ = substrate utilization rate, mass volume^{-1} time^{-1}

b = biomass decay coefficient, time^{-1}

The growth yield coefficient, a_e, was estimated from thermodynamic considerations as follows:

$$a_e = \frac{1}{1 + A} \tag{2.7f}$$

$$A = -\frac{\Delta G_s}{k\Delta G_r} \tag{2.7g}$$

The term A is defined as the electron equivalents of energy derived from the electron donor per electron equivalent of cells synthesized. ΔG_s is the free energy necessary for synthesizing an electron equivalent of cells, whereas, ΔG_r is the free energy released per electron equivalent of substrate oxidized. The term k is related to the efficiencies of electron transfer from the electron donor to acceptor. The free energy for cell sythesis is composed of three terms.

$$\Delta G_s = \Delta G_p/k^m + \Delta G_c + \Delta G_n/k \tag{2.7h}$$

ΔG_p is defined as the free energy necessary for converting the carbon used in cell material to an intermediate level. It is calculated by subtracting the half reaction involving pyruvate from the half reaction of the initial substrate. ΔG_c is the free energy necessary to convert the intermediate level carbon and ammonia into cellular material. This value has been estimated as 7.5 kcal including energy losses during electron transfer. The term ΔG_n represents the free energy needed to produce ammonia from inorganic nitrogen. ΔG_n is zero if ammonia is used, but is 4.17 kcal for NO_3^-, 3.25 for NO_2^- and 3.78 for N_2. The k coefficients are electron transfer efficiencies which average about 0.6 and range between 0.4 and 0.8. The exponent m is equal to +1 if ΔG_p is positive and -1 if ΔG_p is negative. Equation 2.7g reduces to the following if ammonia is the assumed nitrogen source.

$$A = -\frac{\Delta G_p/k^m + 7.5}{k\Delta G_r} \tag{2.7i}$$

Substituting A into equations 2.7f and 2.7e, the cell yield coefficients and substrate utilization rates can be expressed as follows:

$$a_e = \left(1 + \frac{\Delta G_p/k^m + 7.5}{k\Delta G_r}\right)^{-1} \tag{2.7j}$$

$$\frac{dF}{dt} = (1 + A)\left(\frac{dX_a}{dt} + bX_a\right) \tag{2.7k}$$

2.8. Culture Studies on Biodegradation

Extensive studies have been performed to assess the response of naturally occurring and man-made organic chemicals in aerobic biological systems. In studies using anaerobic environments, however, the body of information has been developed only within the past decade. Most involve microorganisms in pure culture or cultured from common anaerobic habitats such as wastewater sludges and lake sediments. Most chemicals of interest have been the class of aromatics with benzoate serving as a model compound.

Microbial degradation of haloaromatic and aromatic compounds has been reported by several authors. Oshima (1965) first demonstrated the ability of nitrate reducers to utilize benzoate. At the time, it was speculated that nitrate was used in a comparable manner to oxygen during aerobic degradation. Oxygen is inserted into the aromatic during hydroxylation and ring cleavage in reactions catalyzed by oxygenase enzymes (Young, 1984). Subsequent studies, however, failed to detect oxygenase enzymes during nitrate reduction involving aromatic compounds, therefore, a separate reductive pathway has been proposed (Williams and Evans, 1975). Using a specie of *Moraxella* isolated from a garden soil, the pathway for benzoate degradation during nitrate reduction was developed. This pathway is shown in Figure 2. Benzoate is initially reduced by the addition of hydrogen followed by a number of proposed but undefined hydrogenation/dehydrogenation reactions before the ring is cleaved forming adipic acid. Taylor, Hearn and Pincus (1979) isolated a specie of *Pseudomonas* which degraded benzoate, o-fluorobenzoate and p-hydroxybenzoate. Using a *Bacillus* specie, Aftring and Taylor (1981) reported that phthalic acid degraded during nitrate respiration. In each of these studies, anaerobic degradation began with removal of the ring substituents resulting in benzoate which degraded according to the reductive pathway described previously.

Bakker (1977) described the degradation of phenol during nitrate reduction in a bacteria culture obtained from a mixture of soil, manure and sewage sludge. This pathway is shown in Figure 3. Bakker reported that phenol, like benzoate, is initially reduced by hydrogenation followed by

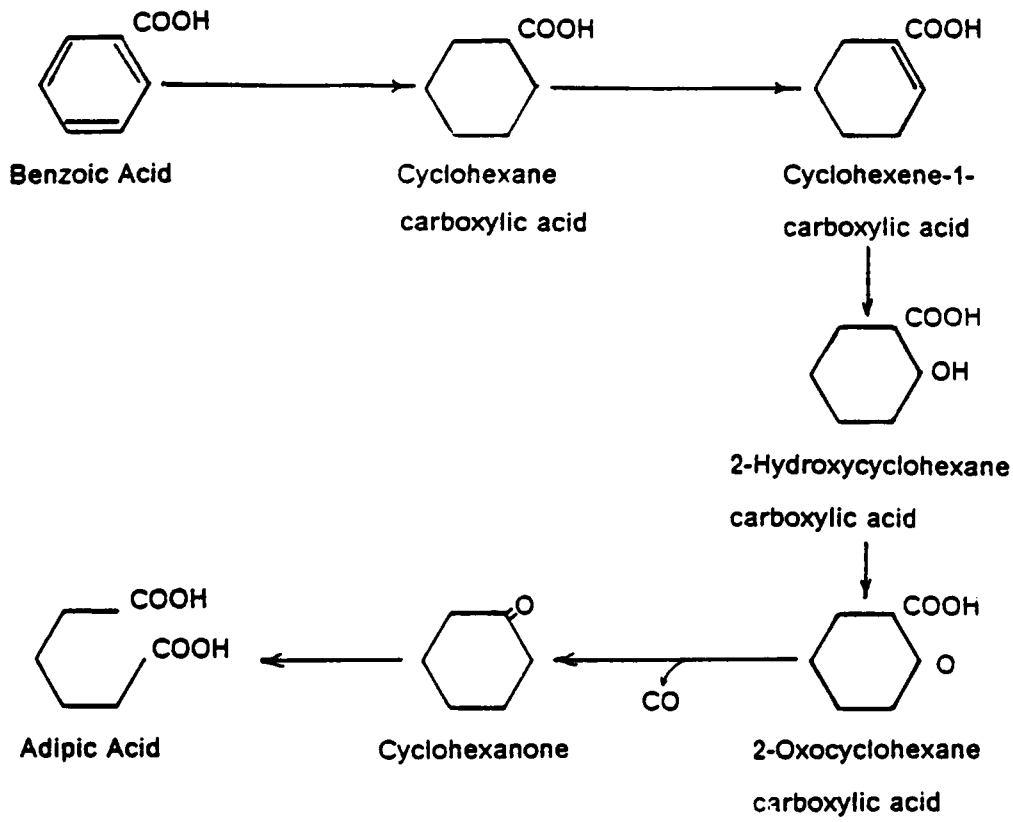

Figure 2. Pathway for benzoate degradation in nitrate reducing conditions (Williams and Evans, 1975).

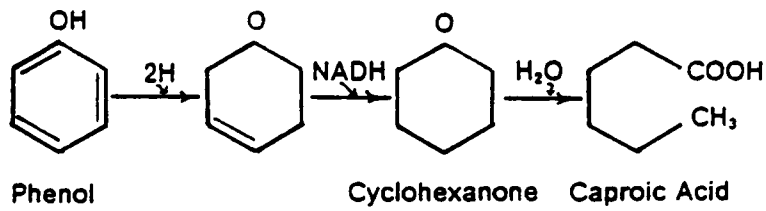

Figure 3. Pathway for phenol degradation in nitrate reducing conditions (Bakker, 1977)

hydrolysis of the ring structure resulting in n-caproic acid. Usable substrates are then produced by β-oxidation. These experiments demonstrated the presence of separate reductive pathways for the degradation of aromatic compounds during denitrification.

In a study of halogenated aliphatic and aromatic compounds in a denitrifying environment, Bouwer and McCarty (1983b) determined that carbon tetrachloride, bromodichloromethane, dibromochloromethane and bromoform biodegraded, however, chlorinated benzenes, naphthalene, chloroform, 1,1,1-trichloroethane and 1,2-dibromoethane did not. The most interesting aspect of these results was that none of the chlorinated benzenes were utilized. The authors speculated that molecular oxygen was necessary for ring cleavage. This would appear to conflict with the results described by others (Williams and Evans, 1975; Evans, 1977). Table 3 lists substrates which have been shown to degrade under denitrifying conditions.

Very little information is available concerning the oxidation of organics during sulfate reduction. Widdel, et al. (1983) determined that acetate, propionate, butyrate, valerate, caproate, benzoate, 4-hydroxybenzoate and phenylacetate were degraded by *Desulfonema magnum* isolated from marine sediments. Ethanol, cyclohexane, carboxylate, adipate, pimelate, glucose, 2-hydroxybenzoate, 3-hydroxybenzoate, urate and nicotinate, however, did not support growth.

Methananogenic bacteria can only utilize a few simple compounds for growth, therefore, they depend on a syntrophic relationship with other organisms to supply those compounds from the degradation of complex organics. Studies on biological degradation of organic compounds under methanogenic conditions, therefore, have used bacterial consortia. For this reason, there has been little concensus in identifying intermediates produced during degradation.

Most early studies of methanogenic biodegradation used benzoate as a model compound. Fina and Fiskin (1960) determined that benzoate was utilized by a culture obtained from bovin rumen producing CH_4 and CO_2 in stoichiometric quantities. They determined that the carboxyl group and carbon 4 were converted to CO_2, while carbon 1 was reduced to methane. Numerous other

Table 3. Substrates for denitrifying biodegradation.

Substrate	Bacteria	Reference
Phenol Benzoate 3-Hydroxybenzoate 3,4-Hydroxybenzoate 4-Hydroxybenzoate o,m,p-Cresol	Enrichment	Bakker, 1977
Benzoate o-Fluorobenzoate p-Hydroxybenzoate	Pseudomonas	Taylor, et al, 1979
Phthalic Acid	Bacillus	Aftring and Taylor, 1981
Carbon tetrachloride Bromodichlormethane Dibromochloromethane Bromoform	Sludge	Bouwer and McCarty, 1983b
Benzoate 2-Aminobenzoate 3-Hydroxybenzoate	Pseudomonas	Braun and Gibson, 1984
p-Cresol	Sediments	Bossert, et al., 1986

studies have been performed to define the pathway of benzoate degradation by methanogenic consortia. The results of these studies have been reviewed by Evans (1977), Young (1984) and Berry, et al. (1987). The summarized pathways for benzoate degradation are shown in Figure 4. Though the pathways are different in terms of their intermediates, each shows ring reduction and cleavage followed by production of aliphatic acids which are converted by β-oxidation to suitable methanogenic substrates. Figure 5 shows the degradation pathway for phenol. The list of substrates amenable to biodegradation in methanogenic conditions has been extended in recent years. A partial list of these chemicals is given in Tables 4 and 5.

Suflita, et al. (1982) reported reductive dehalogenation as the initial step in the biodegradation of halogenated benzoate. Using a bacterial enrichment obtained from sewage sludge, dehalogenation and subsequent mineralization resulted in the production of CH_4 and CO_2. The reaction rate depended on the type of halogen and its ring position but not the number of halogens. A longer lag time was observed for chlor- and fluoro- benzoates than for bromo- and iodo- substituted compounds. In addition, the meta- substituted compounds were degraded faster than the ortho- and para- isomers for both the single and multiple halogenated chemicals.

In a study of monosubstituted phenol degradation by dilute anaerobic sludge, Boyd, et al. (1983) and Boyd and Shelton (1984) determined that the ortho-, meta- and para- isomers of chlorophenol, methoxyphenol, methylphenol and nitrophenol were mineralized with the production of CH_4 and CO_2. During an 8 week incubation period, only p-chlorophenol and o-methylphenol did not degrade significantly. For the monochlorophenols, the rate of disappearance was as follows: ortho- > meta- > para-. In a study of dichlorophenols, reductive dechlorination involving the ortho chlorine to hydroxy group resulted in an accumulation of the resulting monochlorphenol which was subsequently degraded.

Bouwer, Rittmann and McCarty (1981) and Bouwer and McCarty (1983 and 1984) determined that trace quantities of chloroform, carbon tetrachloride, 1,1,1-trichloroethane, 1,1,2,2-tetrachloroethane and tetrachloroethylene biodegraded in methanogenic batch cultures and

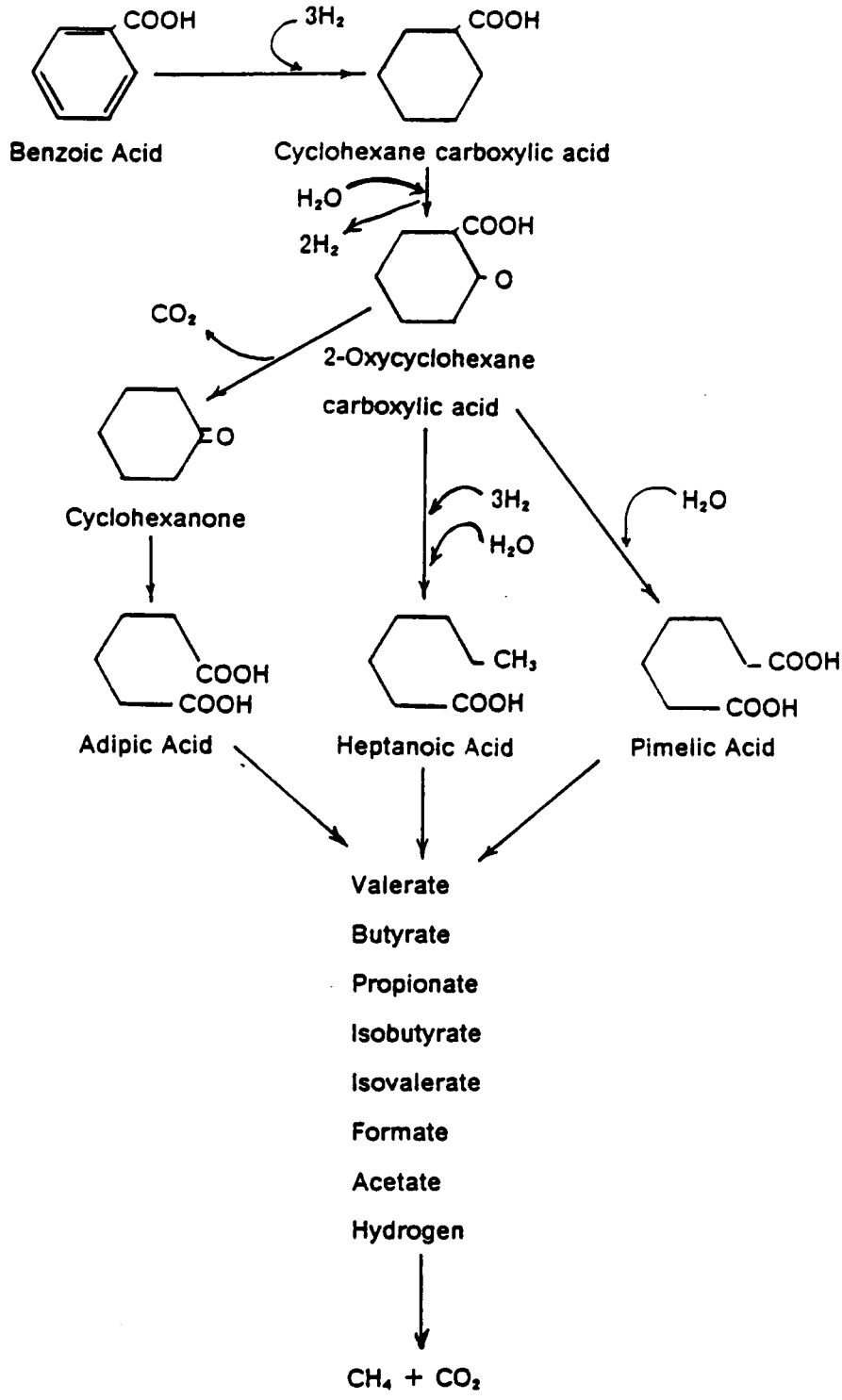

Figure 4. Proposed pathway for benzoate degradation under methanogenic conditions (Evans, 1977)

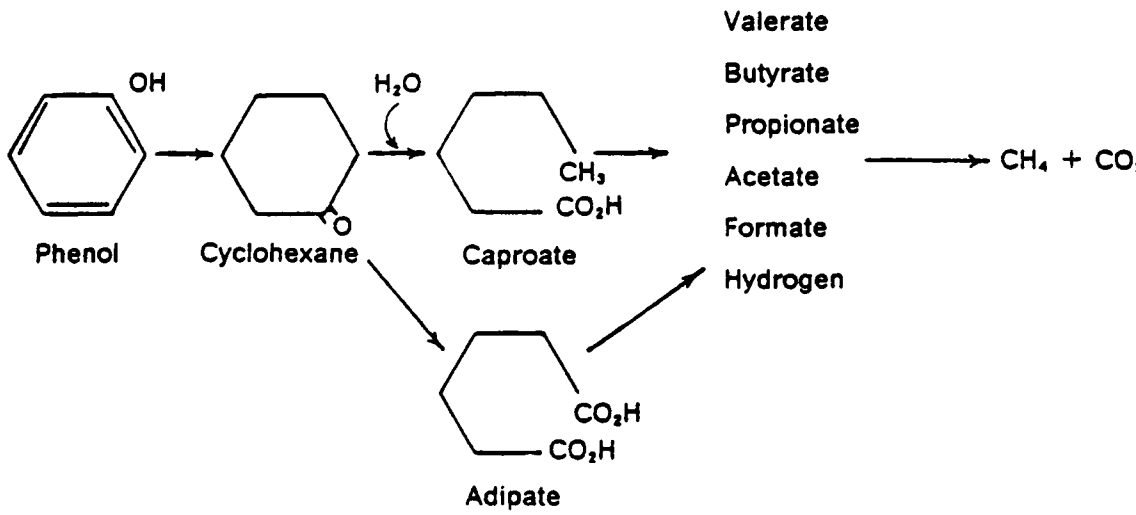

Figure 5. Proposed pathway for phenol degradation under methanogenic conditions (Evans, 1977)

Table 4. Substrates for methanogenic biodegradation.

Substrate	Bacteria	Reference
Benzoate	Bovin Rumen	Fina and Fiskin, 1966
Phenol Catechol	Sludge	Healy and Young, 1978
Vanillin Vanillic acid Ferulic acid Cinnamic acid Benzoic acid Catechol Protocatachuic acid Phenol p-Hydroxybenzoic acid Syringic acid Syringaldehyde	Sludge	Healy and Young, 1979
Ferulic acid	Sludge	Healy, et al., 1980
Chloroform Carbon tetrachloride 1,1,1-Trichloroethane Tetrachloroethylene Bromoform Bromodichloromethane Dibromochloromethane	Sludge	Bouwer and McCarty, 1983c Bouwer, Rittmenn and McCarty, 1981
3-Chlorobenzoate 2 or 4-Iodobenzoate 3-Iodobenzoate 2 or 4-Bromobenzoate 3-Bromobenzoate 3,5-Dichlorobenzoate	Sludge	Suflita, et al., 1982
Syringic acid 2,6-Dimethoxxyphenol Gallic acid Pyrogallol Vanillic acid Veratic acid	Sediments	Kaiser and Hanselmann, 1982
Coniferyl alcohol	Sludge	Grbic-Galic, 1983

Table 5. Substrates for methanogenic biodegradation (con't).

Substrate	Bacteria	Reference
o,m,p-Chlorophenol m,p-Cresol o,m,p-Nitrophenol 2,3-Dichlorophenol 2,4-Dichlorophenol 2,5-Dichlorophenol 2,6-Dichlorophenol 3,4-Dichloropehnol 3,5-Dichlorophenol Phenol	Sludge	Boyd, et al., 1983 Boyd and Shelton, 1984
Phenol Phloroglucinol Hydroquinone p-Cresol	Sludge	Young and Rivera, 1985
2-Propanol 2-Butanol 1,3-Butanediol	Methanospirillum	Widdel, 1986
2-Propanol 2-Butanol Ethanol 1-Propanol	Methanogenium	Widdel, 1986

continuous flow fixed film columns. Acetate was included in the inoculum as the primary substrate. Brominated aliphatics such as bromoform, bromodichloromethane and dibromochloromethane were degraded by a combination of chemical and biological processes. Trichloroethylene, 1,2-dicloroethane, chlorinated benzenes, ethylbenzene, styrene and naphthalene were not removed.

Bouwer, et al. (1986) determined that the degradation of some halogenated aliphatic compounds was influenced by the type of anoxic electron acceptor condition. This study used fixed film continuous flow columns with glass beads inserted to simulate an aquifer matrix. Mixed bacterial cultures were obtained from primary sewage effluent. Anoxic conditions were produced by attaching two columns in series. The influent to the first column contained growth media of acetate resulting in an effluent with a dissolved oxygen concentration of less than 0.5 mg/l. The effluent of the first column then became the influent of the second column. Molybdate was added to the denitrifying column (0.25 mM) and the methanogenic column (1.5 mM) to inhibit growth of sulfate reducing bacteria. 2-Bromoethanesulfanic acid (0.5 mM) was added to the sulfate reducing column to inhibit methanogenesis. Tetrachloroethylene, chloroform, 1,1,2,2-tetrachloroehtane, 1,1,1-trichloroethane, carbon tetrachloride, dibromochloromethane, bromodichlormethane, dibromochloropropane, bromoform and ethylene dibromide were biodegraded in strictly methanogenic conditions. Only 1,2-dichloroethane persisted. In sulfate reducing and denitrifying conditions, chloroform and tetrachloroethylene were not degraded. Ethylene dibromide, 1,1,1-trichloroethane, bromodichloromethane, dibromochloropropane, carbon tetrachloride and bromoform were biodegraded.

2.9. Transport and Fate of Organic Pollutants

Organic chemicals in the subsurface are subject to transport with the groundwater flow. This movement is affected primarily by sorption to aquifer material and biological degradation. Each of these processes must be understood before rational decisions can be made to protect groundwater supplies. Sorption may be a significant removal process for attenuating the movement of organic

chemicals in the subsurface. Karickhoff, et al. (1979) determined that the sorption isotherms for aromatic and chlorinated hydrocarbons were linear in the soluble concentration range of each compound. The linear partition coefficient (K_p) which represents the ratio of the sorbed concentration and the aqueous concentration was independent of the sediment concentration and ionic strength. There was, however, a direct relationship between K_p, the organic carbon content of the soil and the octanol/water partition coefficient. When normalized for organic content, the sand fraction was less efficient than the fine fraction in adsorbing organic compounds. Organic compound mixtures sorbed independently within the linear portion of their sorption isotherm.

Schwarzenbach and Westall (1981) determined that the linear partition coefficient for nonpolar compounds and the organic carbon concentration of the soil were correlated if the organic content was greater than 1 percent. K_p was small in soils with low organic content even if the soil had a high surface area. This study also demonstrated that the partition coefficient could be estimated from the octanol/water partition coefficient in soils with greater than 1 percent organic carbon concentration.

Biodegradation of anthropogenic compounds may provide the most promising solution to the problem of groundwater contamination. Only within the last few years have researchers attempted to characterize the subsurface biodegradation potential and explain the processes which control the fate of organic compounds in groundwater. McCarty and his coworkers have attempted to model biodegradation in the subsurface as a biofilm reactor. The subsurface represents an environment which has a large surface area per unit volume and in many cases low nutrient conditions supporting attached growth. The biofilm model, therefore, may be valid (Rittmann, McCarty and Roberts, 1980). According to Bouwer and McCarty (1984), the biofilm model consists of four processes.

1. Substrate transport into the biofilm from the bulk liquid
2. Substrate utilization with associated bacterial growth following Monod-type kinetics
3. Substrate diffusion through the biofilm according to Fick's Law

4. Biofilm growth and decay

An interesting aspect of the biofilm model is the concept of a minimum substrate concentration (S_{min}) below which no degradation will occur (Rittmann, McCarty and Roberts, 1980; McCarty, Reinhard and Rittmann, 1981; Kobayashi and Rittmann, 1982; Bouwer and McCarty, 1984). S_{min} is defined as the concentration below which bacteria cannot obtain enough energy from utilization to support maintenance requirements. If valid, this concept would have significant effects on the ability of soil bacteria to degrade trace organic chemicals. Utilization of a compound below S_{min} may be possible if the limiting compound is used simultaneously with another more abundant compound which supports the energy requirements of the organism. This process has been termed secondary utilization. The limiting substrate is called the secondary substrate and the substrate which supports growth is called the primary substrate. Using the biofilm model, Bouwer and McCarty (1984) reported that chlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene were biodegraded in aerobic conditions but were persistent in a methanogenic environment. On the other hand, halogenated aliphatics such as chloroform, carbon tetrachloride and 1,1,1-trichloroethane were removed under methanogenic conditions but not in aerobic conditions. These experiments were conducted in a continuous flow column using glass beads to simulate the aquifer matrix. A mixed culture bacterial population was added and acetate was contained in the feed solution as a primary substrate to stimulate secondary utilization. While these experiments demonstrated the degradability of certain xenobiotic compounds, the relevance to actual subsurface conditions is uncertain.

Simkins and Alexander (1984) reported that the degradation response observed in mineralization of organic compounds was a function of bacterial population size and substrate concentration. The degradation rates of benzoate ranging in initial concentration between 10 ng/ml and 100 μg/ml were measured using *Pseudomonas* cultures. The data was then fit by nonlinear regression analysis to six models which were derived form Monod kinetics. As a result, Figure 6 was developed which established kinetic regimes whose boundaries were defined only by the initial cell concentration and the initial substrate concentration. According to the authors, the zero order, Monod-no growth and

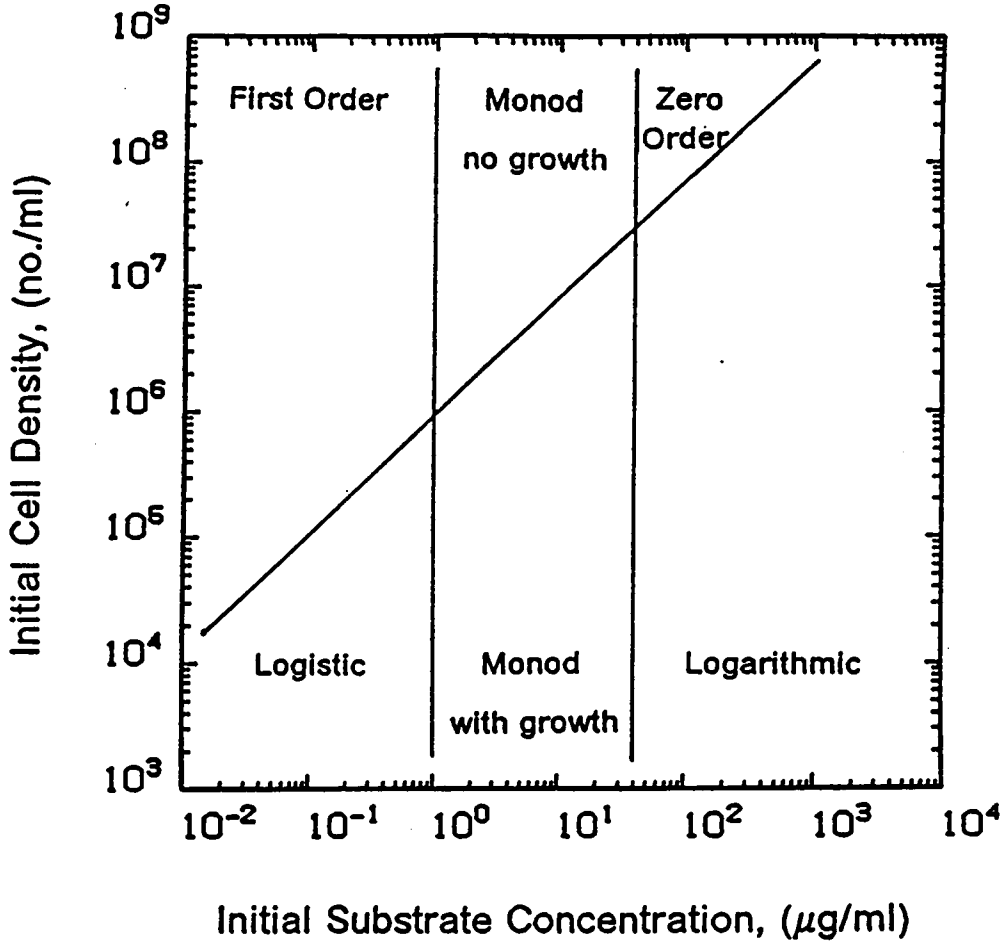

Figure 6. **Kinetic models as a function of initial substrate concentration and initial cell concentration (after Simkins and Alexander, 1984).**

first order kinetic regimes can be approximated from the general Monod equation when the population density is much greater than the initial concentration. The cell yield during substrate utilization is insignificant compared to the initial cell density. In addition, the zero order zone requires that initial concentration be much greater than the half saturation constant (K_s) while the first order regime assumes that the initial substrate is much less than K_s.

In the logarithmic, Monod (with growth) and logistic models, the growth of the population during mineralization is assumed to be significant. The logistic and logarithmic cases reflect the extreme substrate conditions where initial concentration is much less than K_s or much greater than K_s, respectively.

Only a very limited amount of information exists relating biodegradation to site specific conditions. In a study of a creosote contaminated aquifer in St. Louis Park, Minnesota, Ehrlich, et al. (1982) determined that phenolic compounds were biodegraded anaerobically. In contaminated wells, methane was detected and methane producing bacteria were present in significant quantities. No methane or methanogenic bacteria were measured in uncontaminated wells. Phenol was degraded to methane in laboratory reactors containing water from the contaminated well. The fate of polynuclear aromatic hydrocarbons detected in the well was not determined. It was suggested, however, that sorption was an important removal mechanism for PAHs.

Wilson, et al. (1983b) determined that toluene and styrene were slowly biodegraded in microcosms containing soil from two previously uncontaminated sites. Chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane did not degrade in either soil and chlorobenzene was utillized in soil from only one site. Each site was sampled in the unsaturated and saturated zones. If a compound degraded in the unsaturated soil of a site, it also degraded in the saturated soil. These results may conflict with those of Bouwer and McCarty (1981) who reported the biodegradation of chloroform and 1,1,1-trichloroethane in a methanogenic biofilm reactor but not in a denitrifying system. The observation that the test compounds were either utilized slowly or not at all was attributed to the oligotrophic nature of the subsurface bacteria.

Goldsmith (1985) and Novak, et al. (1985) examined the degradation of two gasoline additives, methanol and tertiary butyl alcohol (TBA), in subsurface samples collected from three previously uncontaminated sites. One of the sites was aerobic while the other two were anoxic. Methanol degraded rapidly at all three sites. TBA, on the other hand, was quickly utilized at only the aerobic site. At the anoxic sites, TBA degraded slowly following zero order kinetics in individual microcosms, but indicated a first-order response with respect to the initial concentration. The presence of benzene, toluene and m-xylene did not affect the degradation rate of the alcohols. In each case, biodegradation in the saturated zone was greater than in the unsaturated zone.

White (1986) determined that TBA would degrade rapidly in aquifer material obtained from a site previously contaminated with that compound. Biodegradation of TBA was accompanied by bacterial growth and could be modeled by the Monod equation. TBA degradation in soil from an uncontaminated site was slow, exhibiting the same response as was observed by Goldsmith (1985). This slow rate could not be modeled adequately by Monod kinetics.

In a study on biodegradation enhancement, Wilson (1986c) determined that the addition of nitrate to soil which did not contain an actively denitrifying bacterial population would inhibit the degradation of methanol due to the buildup of nitrite. This inhibition was relieved if the pH was raised above pH 6. The addition of sulfate inhibited methanol and TBA degradation at the site studied. Manipulation of the pH did not affect this condition. Variation of pH alone and the addition of organic substrates did not affect the rate of TBA degradation.

Suflita and Miller (1985) found that chlorophenolic compounds degraded in soil from an actively methanogenic site. The chlorinated compounds were degraded via reductive dehalogenation to phenol which was subsequently mineralized. In nonmethanogenic soil, however, reductive dehalogenation did not occur resulting in a persistence of the chlorophenols. Phenol degraded in both soils. The nonmethanogenic soil was characterized by a gray to black color and slight odor of sulfide which led the authors to assume that active sulfate reduction occurred. These results suggested that reductive dehalogenation was slowed or inhibited by nonmethanogenic, possibly

sulfate reducing conditions. In a subsequent study, Gibson and Suflita (1986) reported that benzoate and phenol were biodegraded in anaerobic microcosms containing either pond sediment, anaerobic digester sludge, methanogenic aquifer material or sulfate reducing aquifer material. Chloroaromatic substrates were degraded in methanogenic aquifer soil but not in sulfate reducing soil. Once sulfate was biologically removed by the addition of acetate, degradation of the chlorinated compounds proceeded. When sulfate was added to the methanogenic aquifer material, the test compounds did not degrade. These results demonstrated that the recalcitrance of a chemical in the subsurface is not necessarily related to a lack of microorganisms capable of biodegradation. Smolenski and Suflita (1987) determined that biodegradation of various cresol isomers was favored in sulfate reducing conditions but was inhibited in methanogenic conditions. Addition of sulfate stimulated degradation, whereas, inhibition of sulfate reduction by the presence of MoO_4^{-2} reduced p-cresol mineralization.

Wilson, et al. (1986a) determined that soil from shallow water table aquifer which had been contaminated with aviation gasoline would degrade benzene, toluene, m-xylene and o-xylene. Soil from the aerobic portion of the aquifer mineralized the test compounds within two weeks, whereas, soil from the anaerobic region required at least 8 weeks. Methane was detected in the headspace of all samples except autoclaved controls.

In a similar study, Wilson, Smith and Rees (1986b) measured the rate of disappearance for five halogenated aliphatic hydrocarbons and four alkylbenzenes in methanogenic aquifer material collected near a sanitary landfill. All test compounds degraded relative to autoclaved controls. The aliphatic compounds, 1,1-dichloroethylene, *trans*-1,2-dichloroethylene and trichloroethylene required long lag times before degradation began. *Cis*-1,2-dichloroethylene and 1,2-dibromoethane were readily consumed. Toluene was the only aromatic tested which did not require a significant acclimation period. Benzene, ethylbenzene and o-xylene degraded after a 20 week lag phase.

Smith and Novak (1987) determined that phenol and four of its chlorinated derivatives were readily degraded in soil from two previously uncontaminated sites. In each case, biodegradation

followed first-order kinetics with the rate of degradation proportional to the initial concentration. The degradation rates did not correlate well with the degree of chlorination. Biodegradation rates were site specific with bacterial population and nitrate concentration cited as important site variations.

Chapter 3

Materials and Methods

3.1. Introduction

The primary objective of this study was to explain the kinetics of TBA subsurface degradation which had been observed by Goldsmith (1985) and White (1986) in microcosms containing soil from Wayland, New York and Dumfries, Virginia. In each of these soils, TBA degraded slowly following a linear degradation pattern in individual microcosms. If the degradation rates were plotted against initial concentration on logarithmic scales, the result was a straight line with a slope of one. In other words, every 10 fold decrease in initial concentration resulted in a 10 fold decrease in degradation rate. The significance of this finding was that low concentrations of TBA would be expected to persist almost indefinitely under natural conditions. This study was undertaken, therefore, in an attempt to explain this kinetic response and describe subsurface conditions which are important in establishing the kinetics.

At the outset of the study, two significant problems were recognized. The first was that there was little information in the literature from which to base a starting point or experimental design.

The second and possibly the most prohibitive was that each microcosm study required an excessive amount of time (in some cases up to a year) before sufficient data could be gathered. This reduced the number of follow-up experiments which could be successfully performed.

The initial experiments were designed to test the importance of three factors on subsurface TBA degradation kinetics. The first was the interaction of bacterial population size and initial concentration as described by Simkin and Alexander (1984). White (1986) suggested that TBA kinetics could be described by Monod (no growth) kinetics within the Simkin and Alexander model over the range of initial concentrations used in his study. In the current study, this conclusion was tested by varying the initial concentration over a wide range in an attempt to induce a different kinetic response which may or may not be consistent with the Simkin and Alexander model.

The second factor which was assumed to be important in determining TBA degradation kinetics was the role of microbial ecology. Previous studies using pure bacterial cultures or enrichments have suggested that the degradation of some organic chemicals is related to the ecology and the type of electron acceptor condition which prevails in the system. To test this, metabolic inhibitors or alternate electron acceptors were added to induce a certain ecological condition or metabolic pathway. Molybdate was added to inhibit sulfate reduction and BESA was added to inhibit methanogenesis. Nitrate was added to stimulate denitrification.

Based on the results of the initial study, additional experiments were performed to test the observed responses on a number of organic chemicals. These substrates were the C1 through C5 alcohols, phenol and DCP. At this point, it was also considered that free energy may or may not have a role in the kinetics of subsurface degradation. A substantial thermodynamic analysis was conducted, therefore, to compare the electron acceptor conditions, hydrogen dependent free energy and thermodynamically predicted cell yield with the degradation rates of the homologous alcohol series and TBA.

In the final stages of the study, experiments were designed to define precisely the role of the metabolic inhibitors by measuring the end products of degradation with and without the inhibitor. In addition, an attempt was made to detect possible TBA degradation intermediates and to stimulate TBA degradation by the addition of hydrogen.

3.2. General Methods

The following general methods were used during the course of this study. All glassware was acid washed in 10% HCl and autoclaved for 20 minutes at 121 °C and 15 psi pressure prior to each use. Distilled water which was used to prepare organic solutions for biodegradation studies and dilution water for bacterial enumeration was autoclaved for 20 minutes at 121 °C and 15 psi pressure. All utensils used in handling the subsurface material were flame sterilized.

3.3. Site Location and Sample Collection

Subsurface material was initially obtained from two previously uncontaminated sites in Blacksburg, Virginia and Newport News, Virginia. The locations of these sites are shown in Figure 7. The Blacksburg site was located on the dairy farm at the Virginia Polytechnic Institute and State University campus. This soil was unsaturated consisting mainly of a tightly packed clay. The Newport News site was located at the Harwood's Mill Water Treatment Plant below the spillway of the Harwood's Mill Resevoir. This soil was saturated just below the surface and was composed primarily of sand and silt. Soil was collected using procedures similar to those developed by Dunlap, et al. (1977) and modified by Wilson, et al. (1983b), Bengstsson (1985) and Novak, et al. (1985). Samples were collected at four to five foot intervals in Shelby tubes driven by a conventional drill rig. For this study, however, soil was used only from a depth of 15 feet for Blacksburg and 4 feet for Newport News. The Shelby tubes were transferred back to the Environmental Engineering Laboratory at Virginia Tech and extruded using a hydraulic jack which pushed the sample

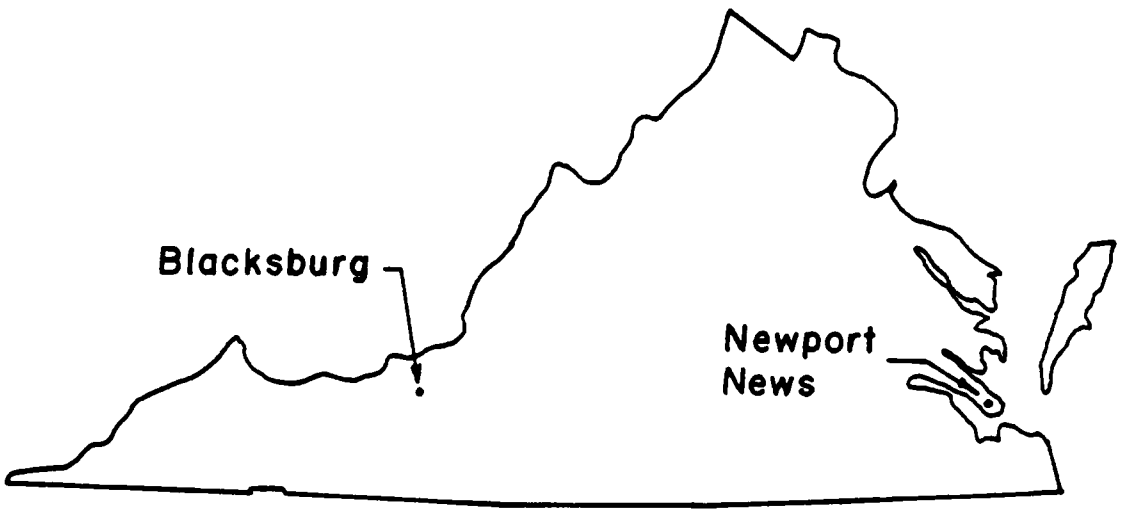

Figure 7. Sampling site locations.

out of the tube. Each soil core was parred using flame sterilized spatulas to remove any material which would be contaminated by contact with the Shelby tube. The sample was then transferred to sterilized mason jars fitted with teflon caps for storage at 10 °C.

Groundwater was collected at the Newport News site by lowering a BOD bottle into the auger hole created by the drill rig. Each sample was fixed by adding nitric acid and stored in ice chests for transport back to the Environmental Engineering Laboratory at Virginia Tech. These samples were used to determine the cation content. Dissolved oxygen was measured at the site by lowering an oxygen probe into the auger hole.

Soil sulfate, nitrate and nitrite were determined using a method described in *Methods of Soil Analysis -- Part 2: Chemical and Microbiological Properties*, (1982). For each sample, 10 grams of soil was mixed with 100 ml distilled water. The resulting solution was shaken for one hour and the anion content of the supernatent was measured using a Dionex Model 2010i Ion Chromatograph equiped with a Dionex HPIC-AS3 column. The eluent was a mixture of sodium bicarbonate (2.8 mM) and sodium carbonate (2.2 mM). The eluent flow rate was 2.0 ml/min. The suppresent was 0.025 N H_2SO_4. Samples for sulfate measurement were extracted with a solution of 100 ppm phosphate as K_2HPO_4 as described by Tabatabai and Bremner (1972). Calcium, magnesium, iron, aluminum, sodium and potassium were determined using a Perkin Elmer Model 713 Atomic Absorption Spectrophotometer. Additional Blacksburg soil was required for the final experiments in this study involving the detection of end products and TBA degradation intermediates. This soil was obtained from a site located about 500 yards from the initial Blacksburg site. Because the drill rig could not be obtained, this soil was collected by digging a trench about four feet deep, then scraping soil from the sides of the trench into sterilized mason jars with a flame sterilized spatula.

3.4. Bacterial Enumeration

The number of bacteria for each soil were determined by the spread plate technique utilizing soil extract media and yeast/peptone extract media. The soil extract solution was prepared by mixing 500 grams of soil in 1 liter of distilled water and autoclaving for one hour. The resulting solution was filtered through Whatman 5, or equivalent filter paper. The filtrate was then autoclaved for 20 minutes and stored at 10 °C until needed. Soil extract media was prepared by adding 100 ml of the concentrated soil extract to 900 ml distilled water. Fifteen grams of Difco agar was added, the pH was adjusted to 7.0 with dilute NaOH and the media was autoclaved for 20 minutes.

Yeast/peptone extract media was prepared by adding 1 gram Bacto-yeast extract, 1 gram Bacto-peptone and 15 grams Difco agar to 1 liter distilled water. The pH was adjusted to 7.0 with dilute NaOH and the solution was autoclaved for 20 minutes.

Spread plates were prepared by pouring 10 to 15 ml of media into petri dishes. The plates were incubated at 20 °C for two days prior to inoculation to ensure the sterility of each media.

The inoculum was prepared by adding approximately 10 grams of soil to 90 ml of sterilized distilled water. The resulting solution was shaken to insure a uniform distribution of soil in the water. This initial mixture was assumed to be a 10^{-1} dilution and was serially diluted to 10^{-6}. One-tenth of a milliliter was pipeted onto a plate and spread with a flame sterilized glass rod. Each dilution was plated in triplicate. The dilution water was also plated to check the sterility. The plates were then incubated at 20 °C for at least 48 hours. After incubation, the number of colonies were counted for the lowest dilution resulting in greater than 30 colonies. The number of bacteria were then determined according to the method described in *Methods of Soil Analysis -- Part 2: Chemical and Microbiological Properties*, (1982).

3.5. Microcosms

Microcosms were constructed of 13 by 100 mm screw-capped test tubes with a 12 mm Teflon-coated septum for sampling to study the biodegradation of the test compounds. Each microcosm contained approximately 5-7 grams of soil. The test compounds were diluted to the desired concentration with sterilized distilled water and introduced as the only carbon source. Each microcosm was mixed once with a vortex mixer. Microcosms of a particular organic concentration were prepared in triplicate. All microcosms were stored in the dark at a constant temperature of 20 °C. To assess the impact of non-biological processes such as adsorption, volatilization and chemical degradation on the loss of substrate, control microcosms were prepared. These contained soil which was autoclaved once a day for five consecutive days at 120 °C and 15 psi pressure. During the first four days, the soil was autoclaved and stored in aluminum foil covered metal containers. The soil was placed in the test tubes and autoclaved a fifth time to kill any bacteria which may have been introduced while loading the microcosms. The static microcosm approach has several advantages: the ability to periodically monitor the samples, the use of native soil microorganisms, a small amount of soil is required for each microcosm which minimized the amount of soil that had to be collected in the field, and the ease in maintaining oxygen limiting conditions. The major disadvantage of static microcosms is that they do not simulate a flowing a groundwater system.

Sodium molybdate (1.0 mM as MoO_4^{-2}) was added in some cases to inhibit sulfate reduction and 5 mM BESA was added to inhibit methanogenesis. Nitrate (0.8-1.6 mM as NO_3^-) was added in some cases to stimulate nitrate reduction and denitrification.

3.6. Analytical Methods

Numerous measurements of the residual organic concentration were performed in order to establish biodegradation rates for each compound in the two soils. Each microcosm was sampled

by removing aqueous samples with a 10 μl syringe. To prevent the introduction of bacteria during the sampling process, the syringe needle was heat sterilized and the septum caps were cleaned with isopropyl alcohol. The septum was covered with wax to prevent the loss of substrate through the hole in the septum created by the syringe needle after sampling. The concentration of each organic was measured by gas-liquid chromatography using a flame ionization detector. A 6′ x 1/8″ stainless steel column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopak C in a Model 5880A Hewlet Packard gas chromatograph was used to identify the alcohols. The carrier gas was nitrogen (24 cc/min) and the sample size was 2 μl. Phenol and DCP were measured using a 2 meter x 2mm glass column packed with 1% SP 1240 DP on 100/120 Supelcoport in a Model 560 Tracor gas chromatograph. The carrier gas was nitrogen (30 cc/min) and the sample size was 2 μl. Each compound was measured isothermally at temperatures selected to provide sufficient detection and separation from the aqueous phase. The following oven temperatures were used: methanol, 80 °C; ethanol, 90 °C; propanol, 110 °C; 1-butanol, 140 °C; pentanol, 150 °C; TBA, 120 °C; phenol, 130 °C; and DCP, 150 °C. The injector port temperature was 150 °C and the detector temperature was 225 °C for each instrument.

Chapter 4

Results and Discussion

4.1. Introduction

The major anions, sulfate, nitrate and nitrite plus selected cations were measured for each soil. These results are presented in Table 6. The major difference in the soils revealed by this analysis was pH. The pH of the Newport News soil was 7.2, while it was 4.2 in the Blacksburg soil (15 feet). The Blacksburg soil consisted mainly of tightly packed unsaturated clay. The Newport News soil was primarily sand which was saturated from water originating from a nearby resevoir.

The primary objective of this study was to explain the kinetic response for TBA degradation observed by Goldsmith (1985) and White (1986) in previously uncontaminated soils from Dumfries, Virginia and Wayland, New York subsurface systems. In these studies, TBA degraded slowly following a zero order rate in individual microcosms. When the utilization rate was compared to the initial concentration, however, it appeared that the zero order rate constant was first order with respect to initial concentration. Some of the microcosms used to obtain these data have been monitored for more than three years without a substantial change in utilization rate. If normal

Table 6. Blacksburg and Newport News soil constituents.

Constituent	Blacksburg	Newport News
Ca^{+2}, mg/l	15.7	30.0
Mg^{+2}, mg/l	1.02	1.52
Na^{+}, mg/l	0.33	4.04
K^{+}, mg/l	2.22	2.68
Fe, mg/l	< 0.03	8.16
Al, mg/l	< 0.10	0.20
SO_4^{-2}, mg/l	6.70	11.10
NO_3^{-}, mg/l	--	--
NO_2^{-}, mg/l	--	--
pH	4.2	7.2

batch kinetics applied, an increased bacterial population should have developed increasing the utilization rate until TBA became limiting. Goldsmith noted that the TBA utilization rates were nearly the same in the Dumfries and New York soils. He speculated, therefore, that anaerobic subsurface systems may respond similarly to TBA. This proposal was supported by the value of the kinetic constants determined for TBA biodegradation in these two soils. The half saturation constant (K_s) was 463 mg/l and the specific substrate utilization rate constant (K) was 0.0018 day^{-1}. The pH of the New York soil was 7.2, while, the pH was 4.5 at Dumfries. Since the rates were nearly identical, microbial cultures must have become adapted to the existing pH so that these were optimum for the indigenous microbial culture.

White (1986) speculated that TBA biodegradation could be described by a Monod-no growth model which has been proposed by Simkins and Alexander (1984). This along with other kinetic models were described in terms of their dependence on initial cell density and initial substrate concentration. These relationships are shown in Figure 6. The Monod-no growth model is based on Michaelis-Menten enzyme kinetics and resembles a linear degradation pattern over an initial substrate range of 1 mg/l to 100 mg/l. Based on the Simkins and Alexander model, White concluded that TBA degradation in the previously uncontaminated soil was due to an enzymatic reaction in which the organism does not receive sufficient energy for growth. Nonspecific extracellular enzymes were also suggested as having a possible role in slow TBA degradation.

Goldsmith and White also reported rapid TBA utilization in soils obtained from Williamsport and Philadelphia, Pennsylvania. In the Williamsport soil, 10 mg/l or less TBA biodegraded within 50 days, whereas, 130 mg/l required about 200 days. Utilization rates were slightly greater in soil obtained from the saturated zone as compared to the unsaturated zone. Kinetic coefficients determined for the saturated zone were as follows: k = 0.6 mg/l/day, K_s = 23.5 mg/l and K = 0.026 day^{-1}. The maximum substrate utilization rate for the unsaturated zone was 0.4 mg/l/day. These results were obtained at 10 °C. Since acridine-orange direct bacterial counts did not vary with depth, it was proposed that the bacteria population in the saturated zone were more active than those in the unsaturated zone.

The Philadelphia subsurface material was obtained from a site previously contaminated with gasoline which contained TBA. TBA biodegradation was rapid following an initial acclimation phase of about 45 days for TBA concentrations of 10 mg/l or less. Measurement of bacterial populations following TBA biodegradation revealed a 10 fold increase, suggesting substrate was being used for growth.

When comparing degradation rates from various studies, it is important to know the method used for calculating substrate utilization. This is especially true for batch systems where the degradation pattern can have several shapes. Erroneous conclusions can be made if the results of a study using one method of calculating rates are compared to studies using different methods. For example, the degradation of many compounds includes an initial acclimation period. If one study includes this lag phase in its calculation of the utilization rate, but another researcher does not, significant discrepancies can result for the same compound. Very few, if any, studies describe the methods for rate calculation. In this study, however, the degradation rates were calculated based on Figure 8.

4.2. Subsurface Bacterial Populations

Significant microbial populations were measured using plate counts with soil extract and yeast extract media for each of the soils used in this study. The choice of media did not affect the results. Spread plates were chosen over the acridine-orange (A-O) direct count method because the Blacksburg soil consisted mainly of clay. It has been found that clay particles are difficult to distinguish from bacteria in a fluorescent sample (White, 1986) so direct counting was not used. In the Blacksburg soil $3.4 \pm 0.2 \times 10^5$ colony forming units (cfu) per gram of soil were measured using yeast extract, while $3.8 \pm 2.0 \times 10^5$ cfu/g soil were counted with soil extract as the growth media. In the Newport News soil, $5.2 \pm 0.2 \times 10^5$ cfu/g soil were present on yeast extract compared to $3.8 \pm 2.0 \times 10^5$ cfu/g soil using soil extract.

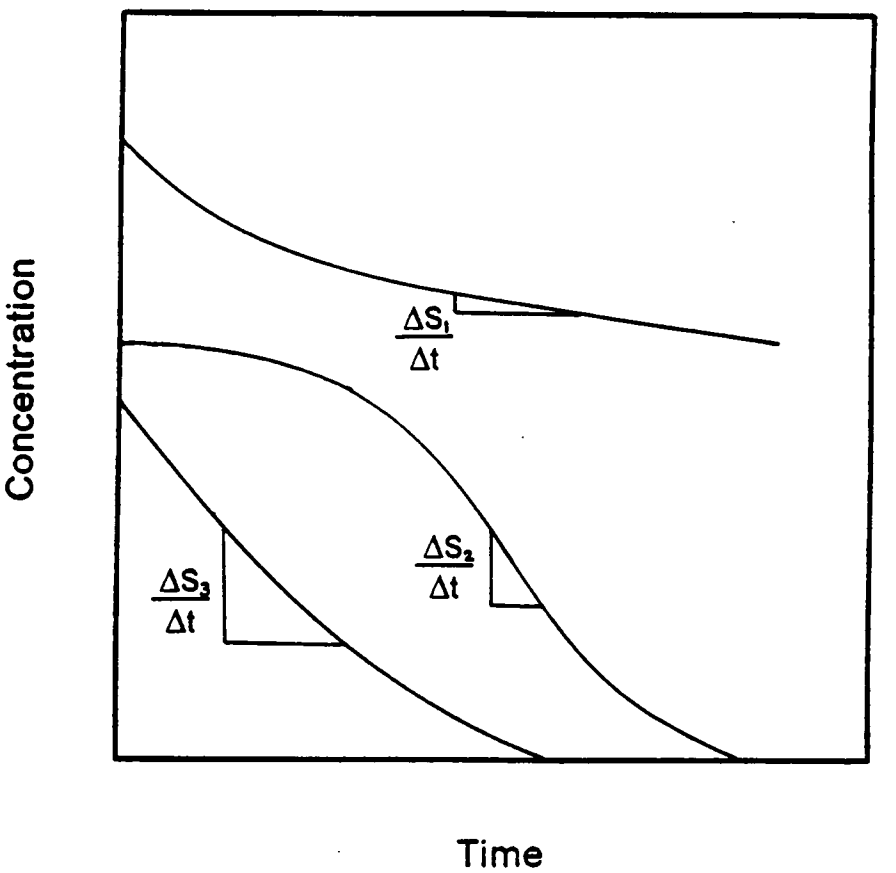

Figure 8. Method for determining biodegradation rates.

The bacterial populations measured for soils in this study were compared to the results using subsurface material collected at other sites for other studies in this lab. These results from these other studies are summarized in Table 7. Goldsmith reported bacterial numbers as a function of depth for subsurface material collected at Williamsport, Wayland and Dumfries. Counts were determined using the acridine-orange direct count method and also using spread plates with soil extract and soil extract amended with formate or methylamine. Only the A-O counts and the soil extract plate counts have been included in Table 7. In general, the A-O counts were consistent throughout the soil profile, whereas, the plate counts varied between one and four orders of magnitude. The A-O results were also consistently higher than the plate counts. Goldsmith reported that the acridine-orange counts were more indicative of the actual bacteria population because of the baises introduced by plate counting media. White measured the bacterial population in a gasoline contaminated soil from Philadelphia, Pennsylvania. For each counting procedure, these results did not vary by more than one order of magnitude throughout the depth. In general, the A-O counts agreed with the plate counts. The bacterial counts observed in the Blacksburg and Newport News soils were comparable to those found at the uncontaminated sites using the plate counting technique. The populations were one to two order of magnitudes less when compared to the contaminated site.

4.3. TBA Degradation

TBA degradation was examined in the Blacksburg and Newport News soils for comparison with results obtained by Goldsmith and White. For the Blacksburg soil, two sets of microcosms were initially constructed. The first set contained TBA concentrations ranging from approximately 200 to 10,000 mg/l and were incubated in an anaerobic glove box. The second set was dosed with about 10 to 5000 mg/l TBA and stored in an incubator not exposed to anaerobic conditions. These two sets of microcosms were made because of concern that anoxic conditions were not maintained in the microcosms. In both sets of microcosms, TBA exhibited the same response as was observed by Goldsmith and White. For the microcosms stored in the anaerobic glove box, TBA degraded

Table 7. Bacteria populations (cfu/g soil) for sites studied by Goldsmith (1985) and White (1986).

Location	Depth (feet)	AO Technique (cfu/g soil)	Soil Extract (cfu/g soil)
Williamsport	0	$5.6 \pm 1.9 \times 10^7$	$3.0 \pm 0.3 \times 10^7$
	12	$3.9 \pm 1.4 \times 10^7$	$3.5 \pm 2.1 \times 10^3$
	30	$4.6 \pm 2.7 \times 10^7$	$1.4 \pm 0.8 \times 10^5$
Wayland	0	$1.0 \pm 0.4 \times 10^8$	$1.0 \pm 0.4 \times 10^6$
	6	$7.6 \pm 3.8 \times 10^7$	$9.3 \pm 1.1 \times 10^5$
	12	$8.0 \pm 6.4 \times 10^7$	$1.1 \pm 0.1 \times 10^5$
Dumfries	0	$1.0 \pm 0.4 \times 10^8$	$9.7 \pm 0.6 \times 10^6$
	11	$7.3 \pm 3.7 \times 10^7$	$< 10^3$
	14	$3.1 \pm 2.2 \times 10^7$	$3.3 \pm 0.4 \times 10^6$
	30	$3.9 \pm 3.4 \times 10^7$	$5.6 \pm 0.1 \times 10^5$
	57	$1.1 \pm 0.6 \times 10^8$	$5.2 \pm 0.9 \times 10^6$
	80	$7.3 \pm 5.6 \times 10^7$	$9.8 \pm 0.5 \times 10^5$
	102	$4.8 \pm 4.5 \times 10^7$	$1.1 \pm 0.3 \times 10^5$
Philadelphia	0	6.8×10^7	4.3×10^7
	25	3.9×10^8	1.1×10^6
	45	3.8×10^7	4.0×10^7

slowly at all concentrations, however, the rate increased as the initial concentration increased (Figure 9). The degradation rate ranged from about 2.5 mg L^{-1} day^{-1} g $soil^{-1}$ at an intial concentration of 9000 mg/l to 0.03 mg L^{-1} day^{-1} g $soil^{-1}$ at an initial concentration of 175 mg/l. Analysis of these samples was stopped after 77 days because there was a noticeable loss of moisture through the septum cap due to holes left by the syringe needle after repeated sampling. All other microcosms used in this study were sealed with wax after each analysis to minimize losses

The TBA degradation pattern for the microcosms stored in the incubator was similar to the pattern for those stored in the anaerobic glove box (Figure 10). The degradation rates ranged from about 0.7 mg L^{-1} day^{-1} g $soil^{-1}$ at an initial concentration of approximately 10,000 mg/l to 0.002 mg L^{-1} day^{-1} g $soil^{-1}$ at an initial concentration of about 20 mg/l. As a result, it was concluded that the degradation occured under anaerobic conditions and the range of TBA concentration over which zero order degradation was observed was at least 4 orders of magnitude.

TBA readily degraded in the Newport News soil. Degradation rates ranged from 0.01 mg L^{-1} day^{-1} g $soil^{-1}$ for microcosms initially dosed with 1 mg/l to about 0.2 mg L^{-1} day^{-1} g $soil^{-1}$ in soil containing 6000 mg/l TBA. Figure 11 shows the degradation pattern of Newport News microcosms containing between 100 mg/l and 6000 mg/l TBA. The degradation rate did not vary significantly in microcosms initially containing 10 mg/l to 6000 mg/l.

4.4. Degradation of Other Test Compounds

The C1 through C5 alcohols, phenol and DCP were added as the sole carbon sources in microcosms containing Blacksburg and Newport News soils. Each of these compounds is used in various industrial applications including solvents, pesticides and gasoline. All are very soluble, therefore, they may migrate far from any point of contamination. 1-Butanol was included to provide a comparison with the response of tertiary butanol. In contrast to TBA, all of these compounds were readily degraded in both soils. Approximately 20 mg/l of the alcohols and 10 mg/l to

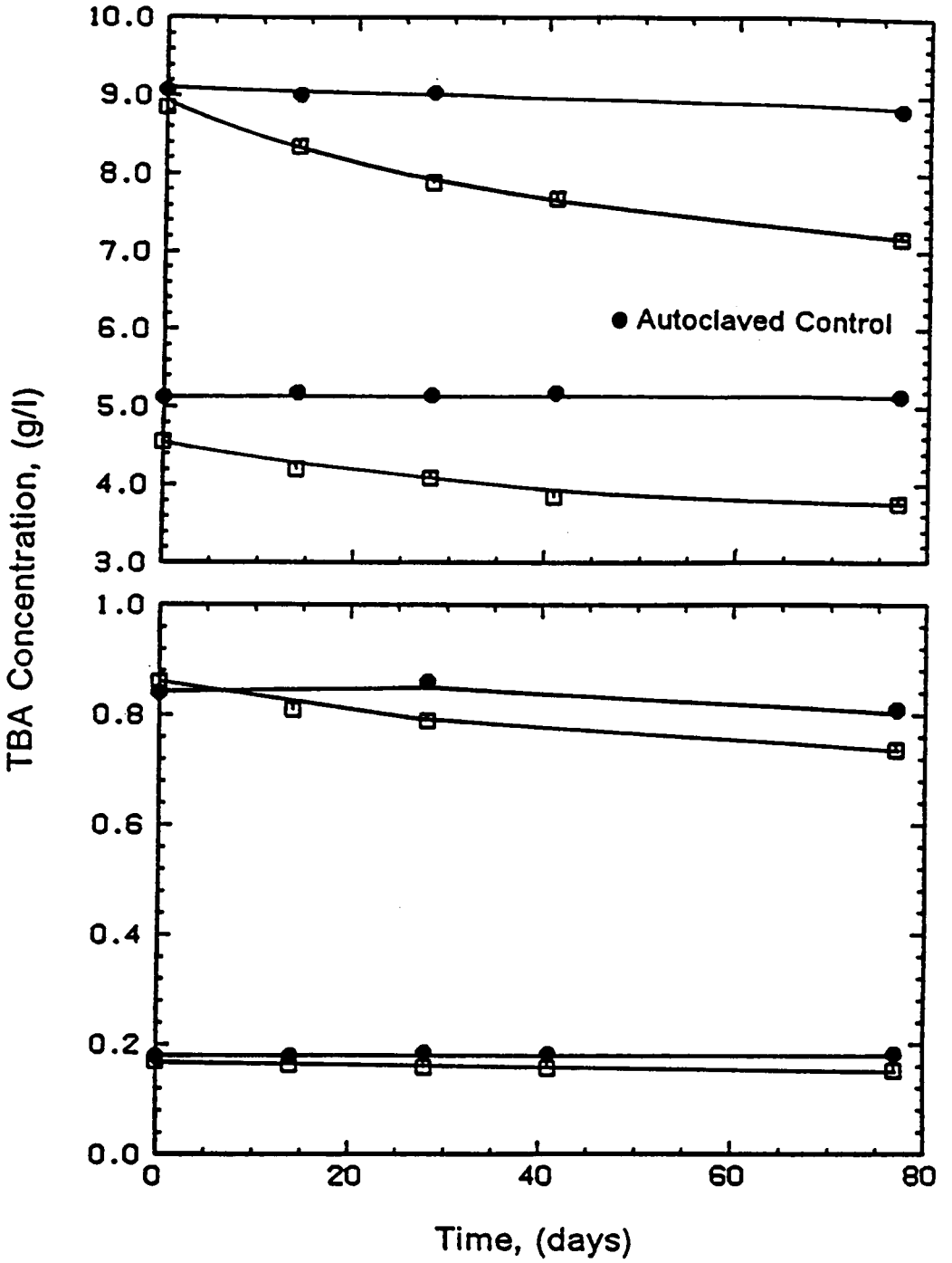

Figure 9. TBA biodegradation in Blacksburg soil (site 1, 15 feet) with various initial concentrations incubated in anaerobic glove box.

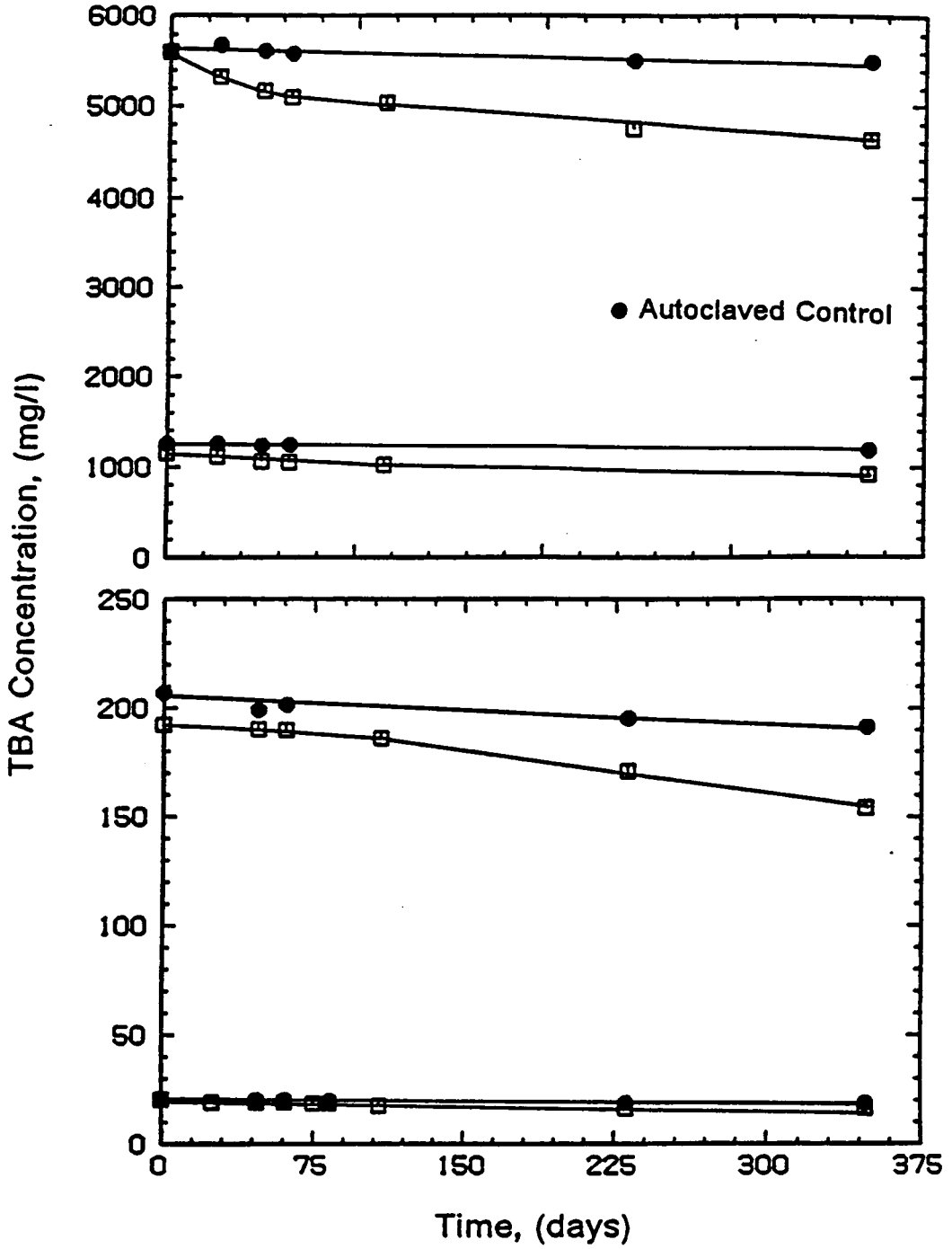

Figure 10. TBA biodegradation in Blacksburg soil (site 1, 15 feet) with various initial concentrations not stored in the anaerobic glove box.

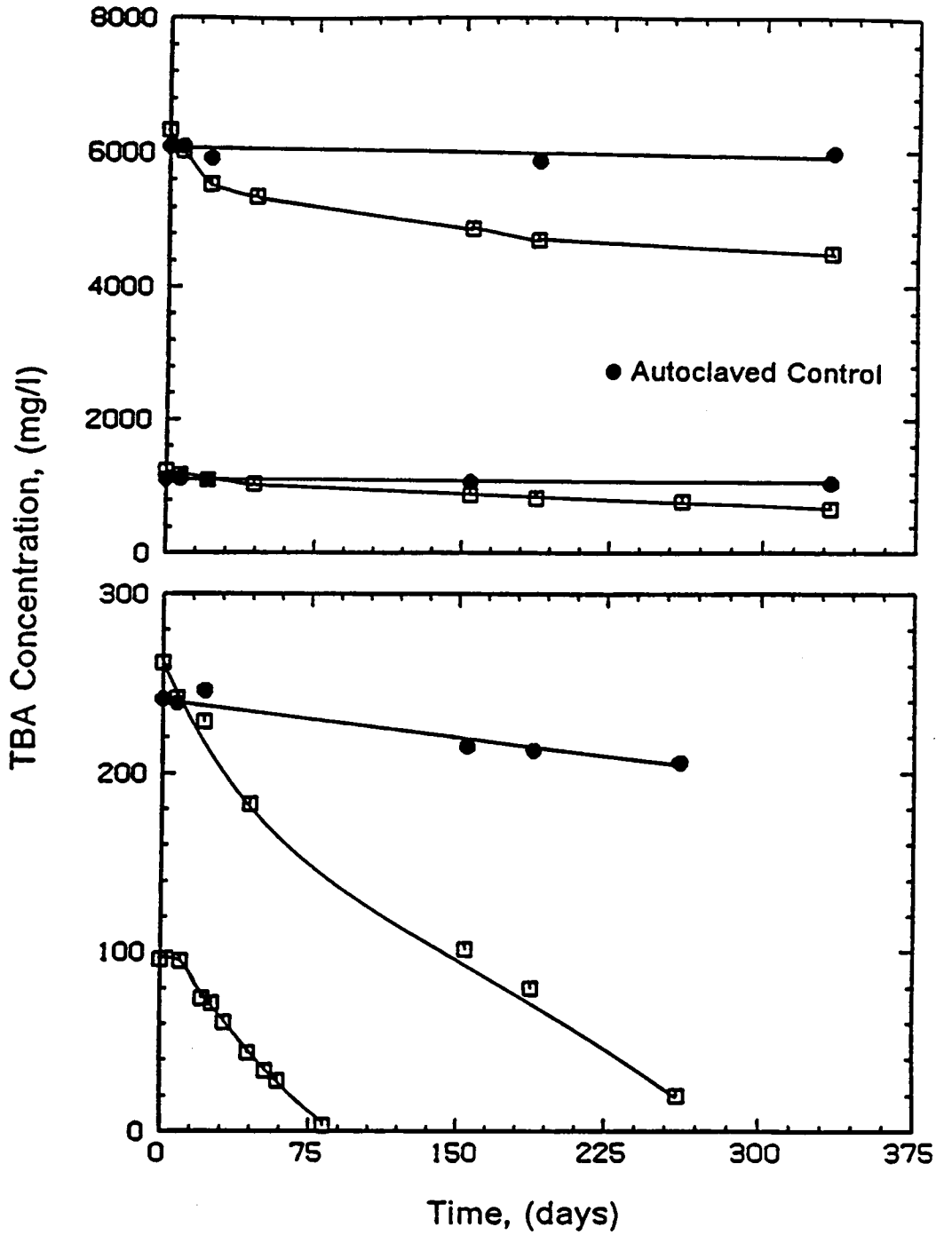

Figure 11. TBA biodegradation in Newport News soil with various initial concentrations.

15 mg/l of the two phenols were degraded below the detection limit within 20 days in the Blacksburg soil (Figures 12 - 14). There was a slight loss of substrate in some of the autoclaved controls possibly due to the presence of organisms which survived the sterilization process or through introduction of organisms during sampling. When compared to non-autoclaved microcosms, however, the loss of substrate was not appreciable in the controls. Substrate loss by adsorption and volatilization, therefore, was considered minimal. There was a 10 day lag period before degradation of methanol occurred and a 5 day lag before degradation of ethanol began. All other compounds degraded without a noticeable acclimation period.

Degradation in the Newport News soil was very rapid for each of the test compounds. Approximately 100 mg/l ethanol, propanol and 1-butanol degraded in 8 days or less while pentanol degraded in 21 days. Methanol was degraded more slowly than the other alcohols requiring about 26 days. One hundred mg/l phenol degraded in about 30 days, whereas, 70 mg/l DCP required about 29 days. No significant substrate loss was observed in the autoclaved controls. These degradation patterns are shown in Figures 15 through 17.

Figure 18 shows the substrate utilization rate for each of the compounds in both soils. The order of the biodegradation rates for the test compounds was as follows: ethanol > methanol > propanol > phenol > 1-butanol > pentanol > > DCP > > TBA for the Blacksburg soil. In the Newport News soil, the utilization rates were as follows: ethanol > propanol > methanol > 1-butanol > pentanol > phenol > TBA > DCP. The degradation rates were substantially greater in the Newport News soil than in the Blacksburg soil. The difference in rate between the two soils ranged from a factor of two for phenol to a 1000 fold difference for TBA. The degradation rate decreased as the chain length increased for the C2 through C5 alcohols. The rate of methanol degradation, however, did not fall into the same pattern exhibited by the other straight chain alcohols. Methanol degraded faster than propanol in the Blacksburg soil but slower than propanol in the Newport News soil.

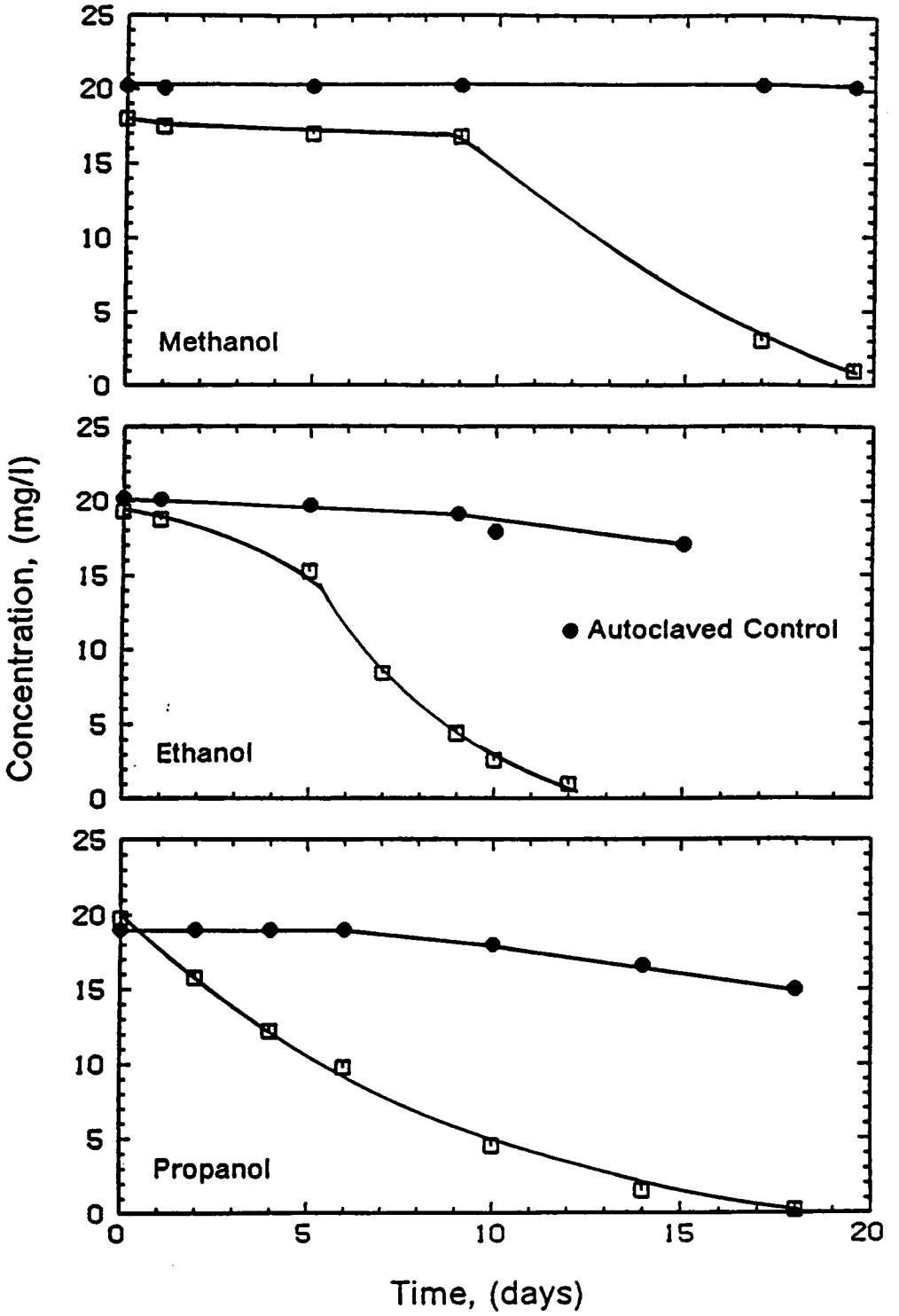

Figure 12. Methanol, ethanol and propanol biodegradation in Blacksburg soil (site 1, 15 feet).

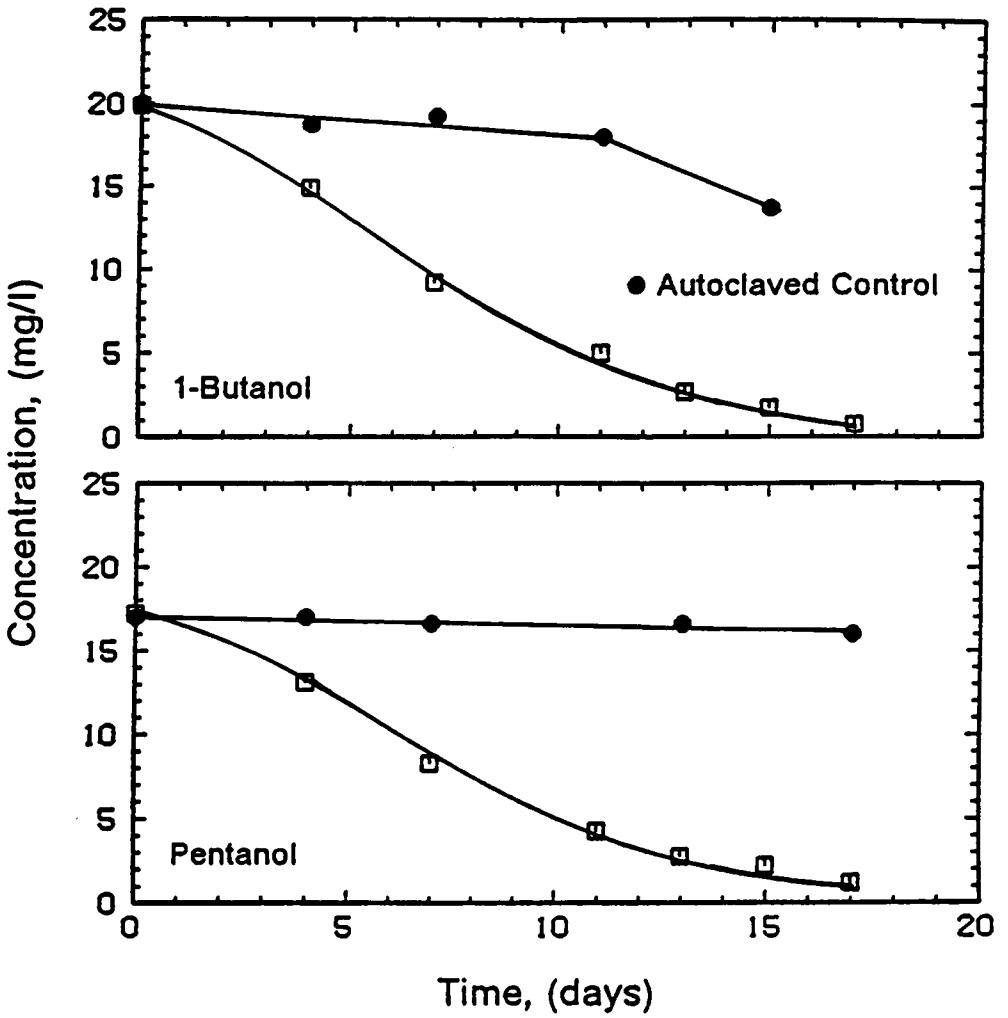

Figure 13. 1-Butanol and pentanol biodegradation in Blacksburg soil (site 1, 15 feet).

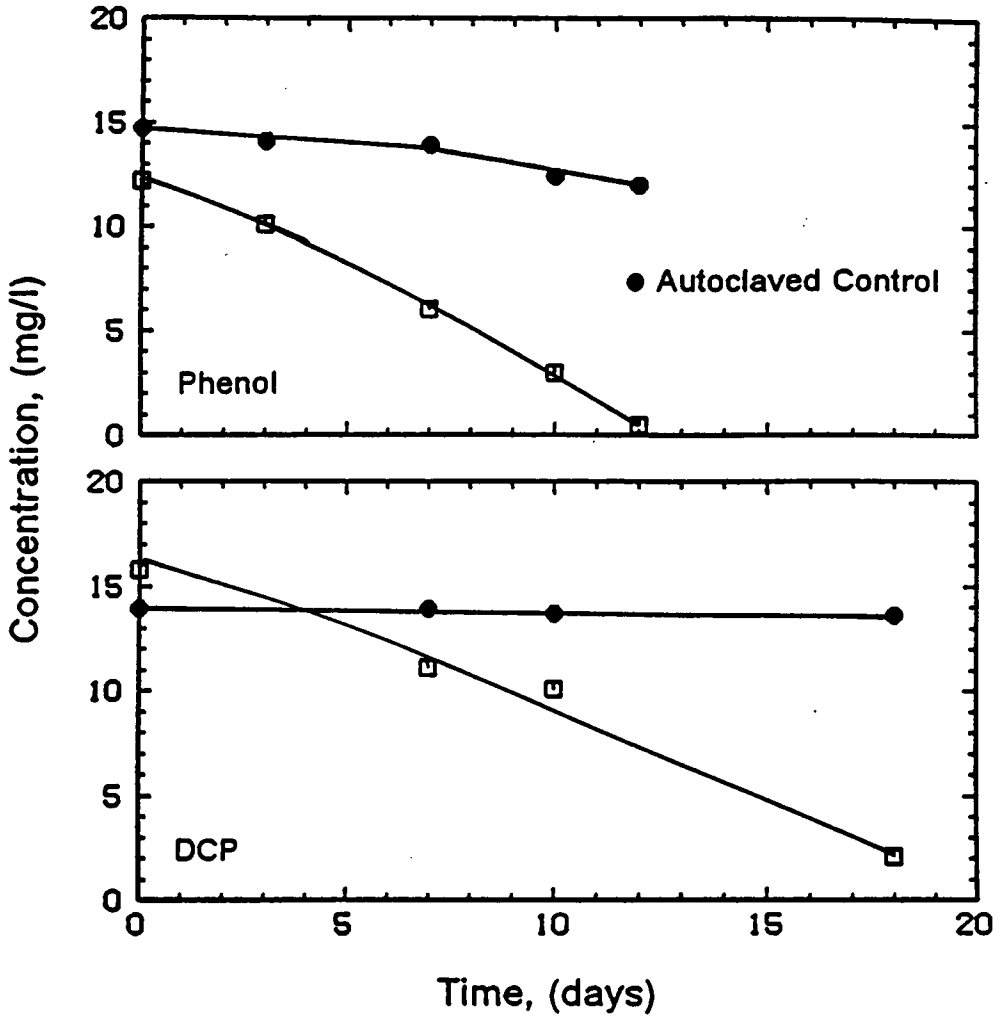

Figure 14. Phenol and DCP biodegradation in Blacksburg soil (site 1, 15 feet).

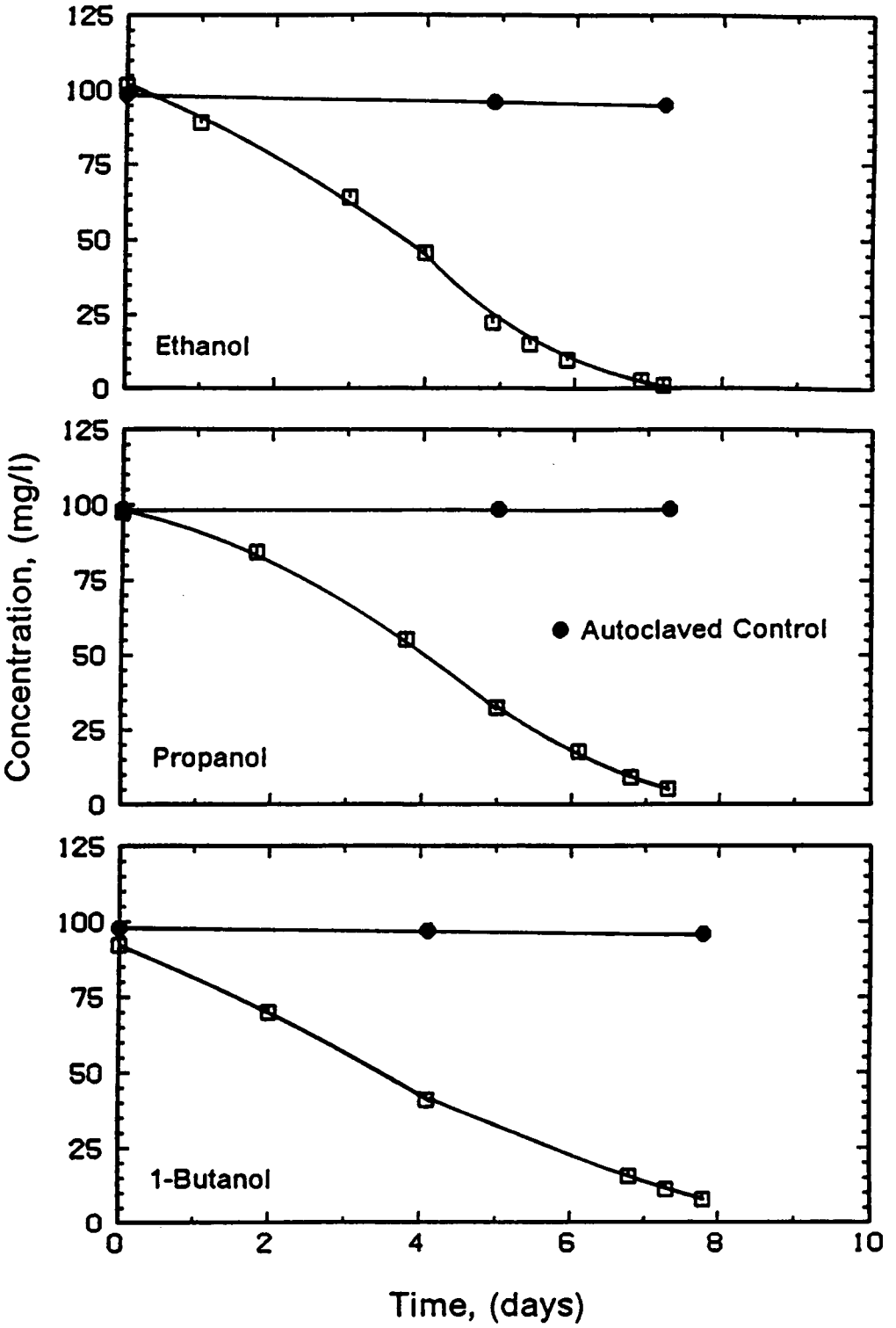

Figure 15. Ethanol, propanol and 1-butanol biodegradation in Newport News soil.

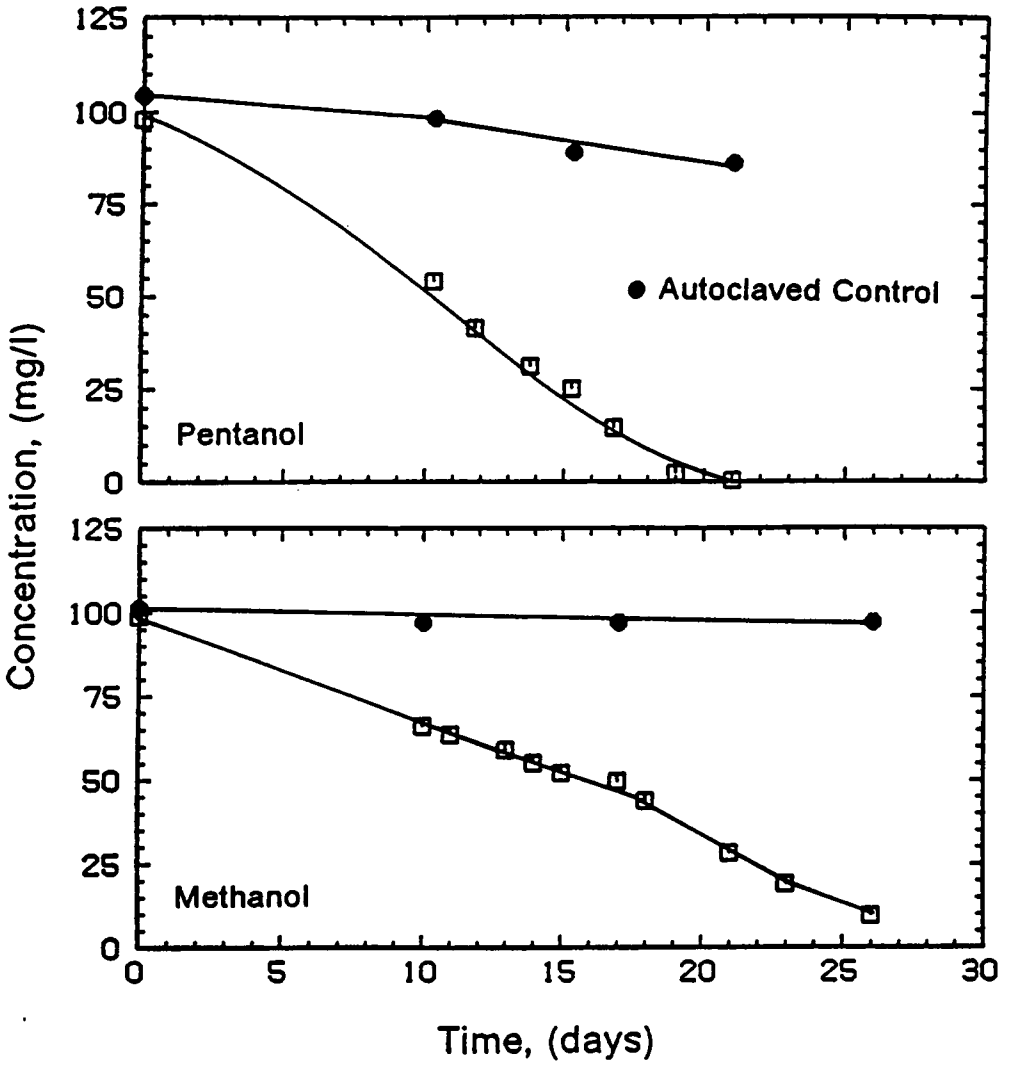

Figure 16. Pentanol and methanol biodegradation in Newport News soil.

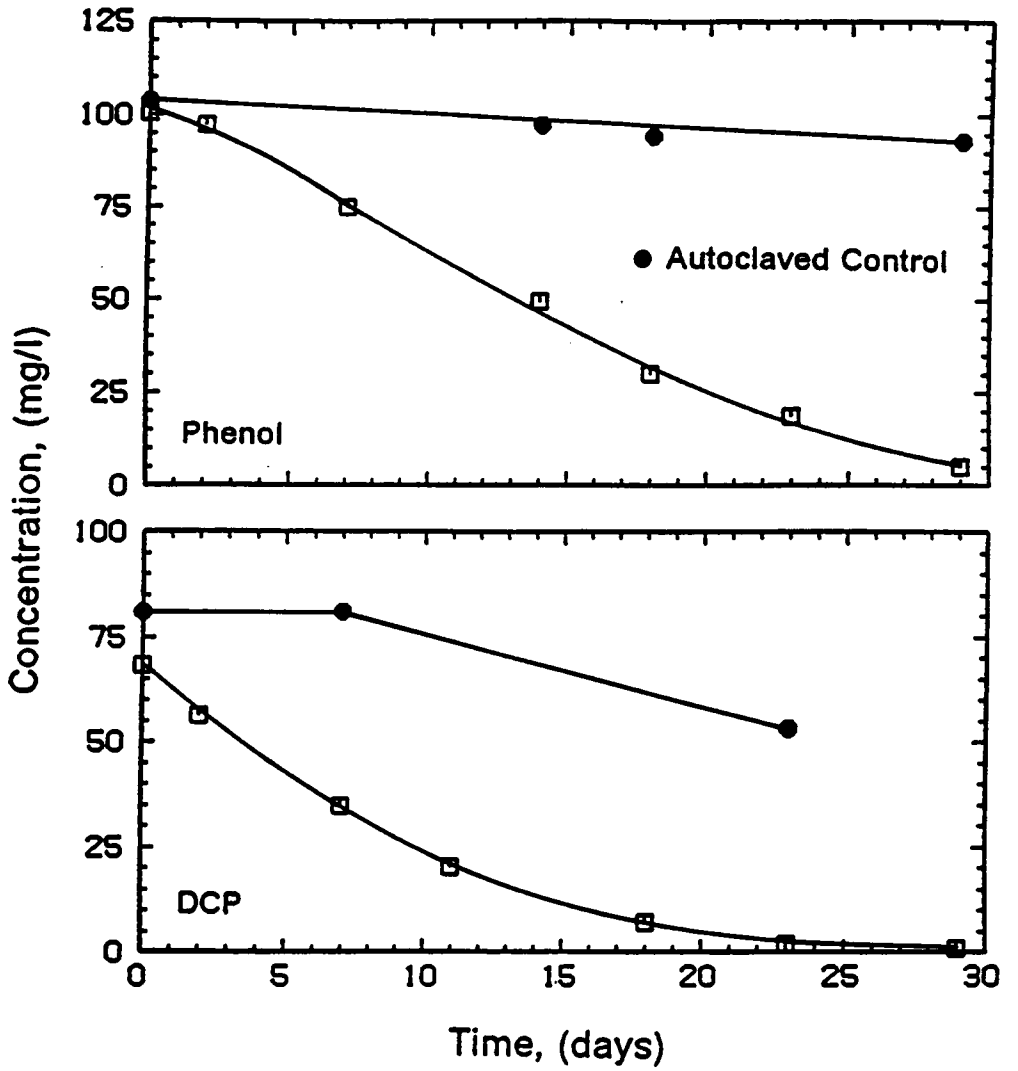

Figure 17. Phenol and DCP biodegradation in Newport News soil.

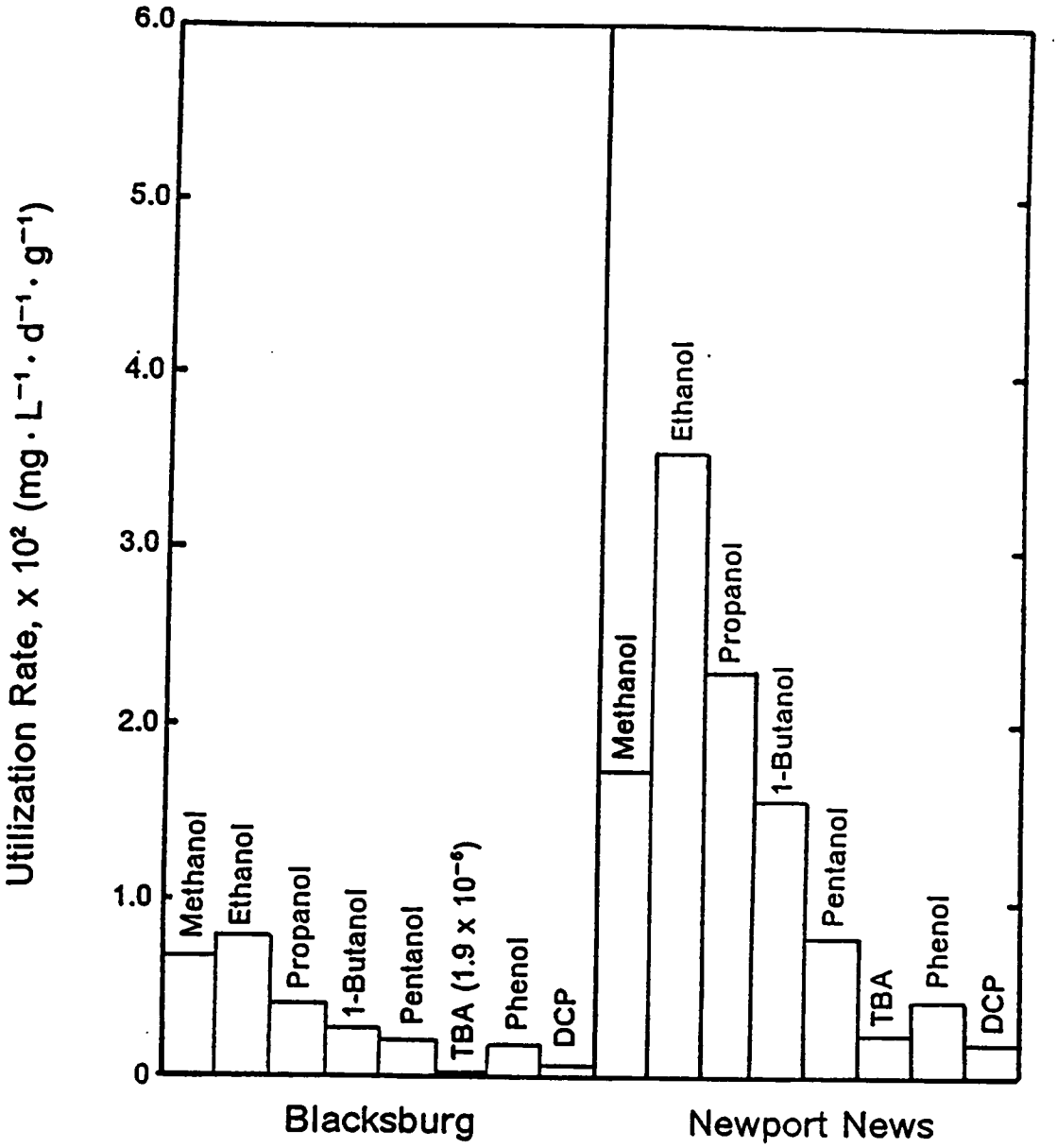

Figure 18. **Biodegradation rates of test compounds in Blacksburg (site 1, 15 feet) and Newport News soils.**

This general degradation rate pattern for the alcohols has also been measured in a study which determined the aerobic biodegradability of various families of aliphatic compounds by activated sludge (Gerhold and Malaney, 1966). In these experiments, oxygen uptake rate was used as a measure of degradation. Ethanol degradation had consumed the highest percentage of the theoretical oxygen demand followed by propanol, 1-butanol, pentanol and methanol after a twenty-four hour incubation period. TBA was only slightly oxidized during the 24 hour test period. The only difference between this series of oxygen utilization rates and the biodegradation rates under the anoxic conditions in this study, is the location of methanol. This is easily explained, however, since the 24 hour oxygen utilization measurement would include any acclimation period, whereas, the degradation rates determined here do not include a lag phase. These results suggest the importance of chemical structure on biodegradability. The more complicated the structure, the more resistent it was to degradation. This was especially true for the two forms of butanol. Tertiary butanol was much more resistent to degradation in the Blacksburg and Newport News soils than was 1-butanol.

4.5. Inhibition Experiments

Some studies in the literature contend that the ability to degrade and the rate at which degradation occurs may depend on competing ecological factors in the subsurface microbial community. Very little, however, is known about these interrelationships. Molybdate and 2-bromoethanesulfonic acid (BESA) were added as metabolic inhibitors of sulfate reduction and methanogenesis, respectively, to determine if ecological factors involving subsurface organisms are important to biodegradation. Any change in response in the presence of an inhibitor was assumed to be attributed to its affect on the ecology of the system and not the chemistry of the system While it is acknowledged that inhibitors are not considered to be 100% specific, evidence presented in the literature suggests that these two inhibitors can be used with some confidence. Molybdate (1 mM) was added to microcosms containing Blacksburg soil dosed with TBA. Microcosms containing 15 mg/l and 5 mg/l TBA plus molybdate degraded to approximately zero within 95 and 100 days, respectively (Figure 19a and b). For microcosms dosed with TBA alone, the degradation rate was

typical of the Blacksburg soil as described previously. Very little degradation occurred over the 240 days in which these microcosms were monitored. These were unexpected results since TBA had always been observed to slowly degrade in this study using Blacksburg soil and in the anoxic uncontaminated subsurface systems examined by others. This experiment, therefore, was repeated using approximately 20 mg/l TBA. As Figure 19c indicates, the same results were observed.

Molybdate was also added to Blacksburg soil microcosms containing the other test compounds to determine if the effect that was observed with TBA was common to the biodegradation of organics in general in this soil. The presence of molybdate increased the rate of substrate utilization. As shown in Figure 20, 18 mg/l methanol was degraded in about 20 days, whereas, a similar concentration of methanol plus 1 mM molybdate degraded in 17 days. In this case, the lag phase was included in the rate calculations since this is the portion of the degradation response which appeared to be most affected by the inhibitor. Likewise, 12 mg/l phenol biodegraded in 7 days in the presence of molybdate but required 12 days without molybdate (Figure 21). The magnitude of the effect varied among substrates. The presence of molybdate affected the phenols more than the alcohols. Figures 22 through 24 compare the degradation rates of all the test compounds with and without molybdate. The presence of molybdate increased the methanol degradation rate 32%, ethanol 14%, propanol 52%, 1-butanol 10%, pentanol 41%, phenol 70%, DCP 307% and TBA 1390%.

BESA was also added to Blacksburg soil microcosms containing each of the test substrates except TBA. In general, the presence of BESA decreased the rate of substrate utilization. Eighteen milligrams per liter methanol required about 25 days to degrade if BESA was included but only 20 days in microcosms containing only methanol (Figure 20). Similarly, 12 mg/l phenol was degraded in 12 days without BESA but required 18 days in microcosms with added BESA (Figure 21). Figures 56-59 in Appendix A show the effect of BESA on the remainder of the test substrates in the Blacksburg soil. Like molybdate, the magnitude of the BESA effect varied with the substrate and appeared to be more pronounced for the phenols. Figures 22 through 24 also show the effect of BESA on degradation rates. The presence of BESA decreased the substrate utilization rate of methanol by 49%, ethanol 11%, propanol 19%, 1-butanol 3%, pentanol 5%, phenol 30% and

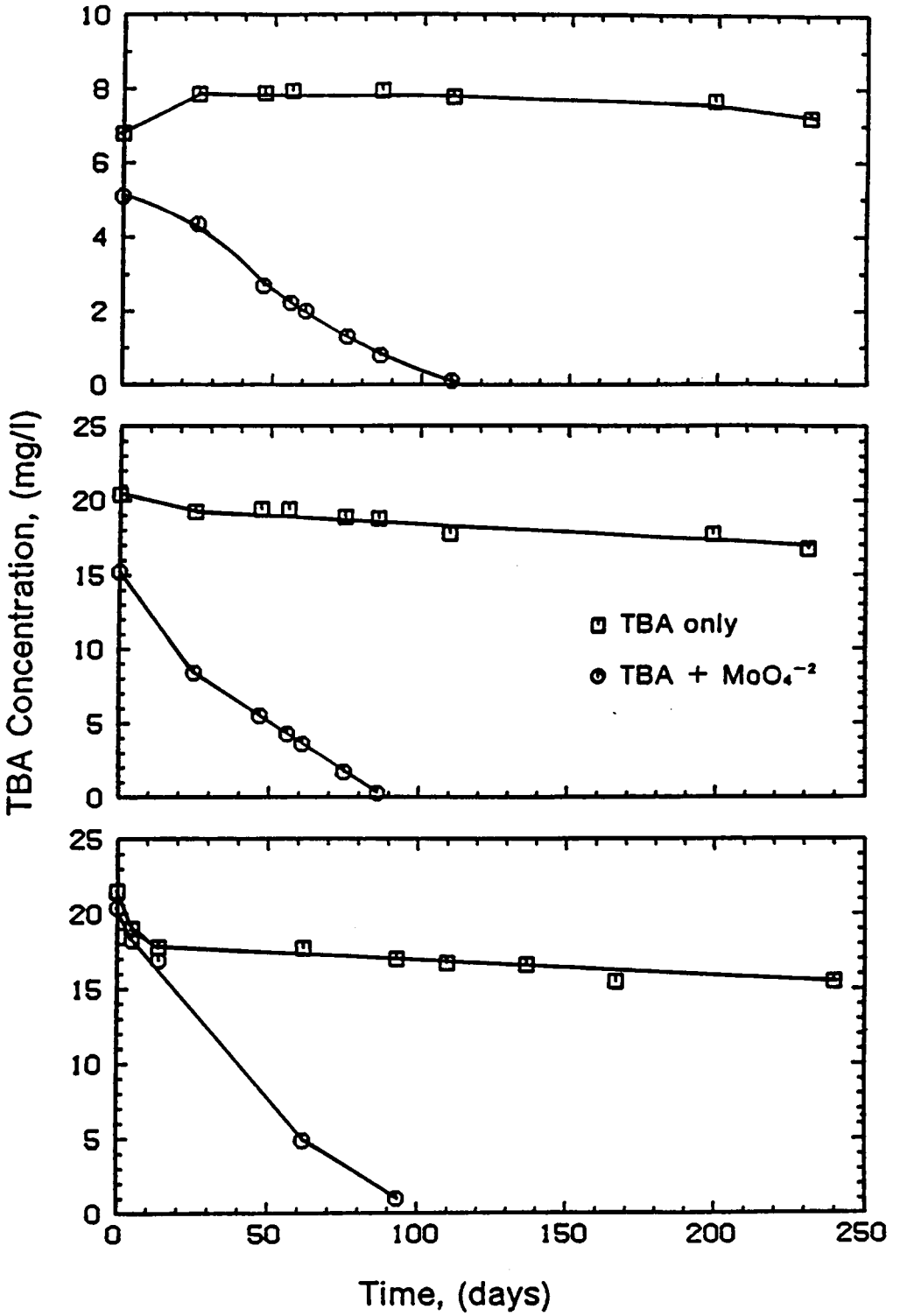

Figure 19. TBA biodegradation in Blacksburg soil (site 1, 15 feet) with and without molybdate (a: Co = 6 mg/l; b: Co = 15 mg/l; c: Co = 22 mg/l)

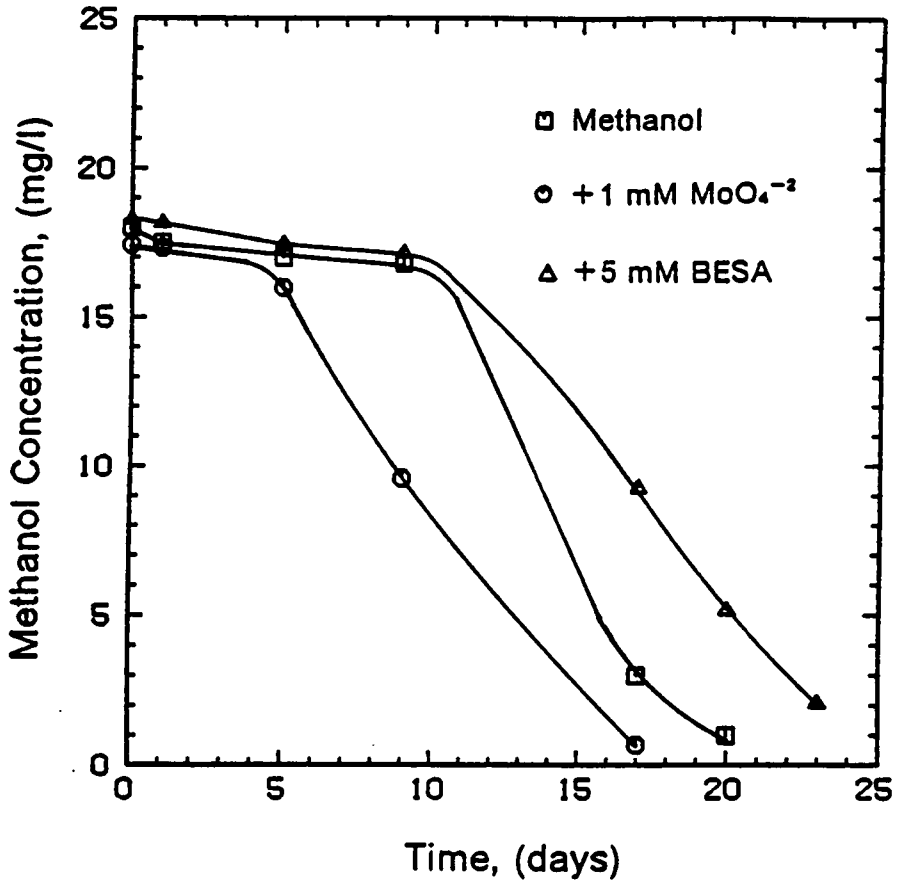

Figure 20. Methanol biodegradation in Blacksburg soil (site 1, 15 feet) with and without molybdate and BESA.

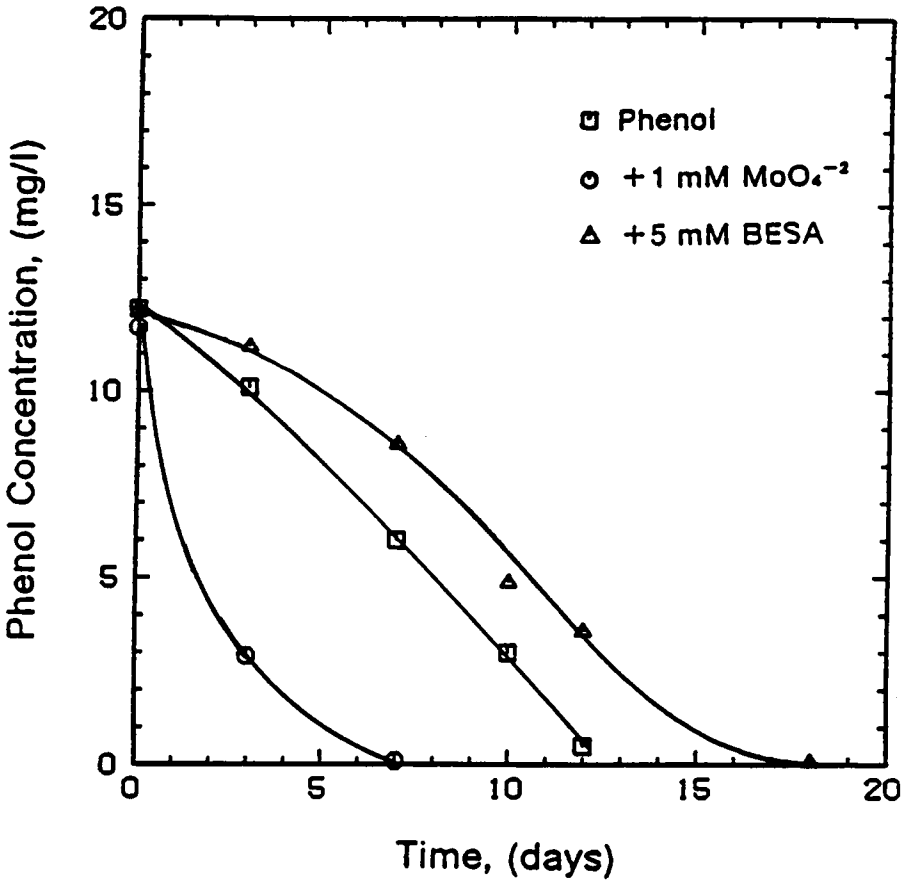

Figure 21. Phenol biodegradation in Blacksburg soil (site 1, 15 feet) with and without molybdate and BESA.

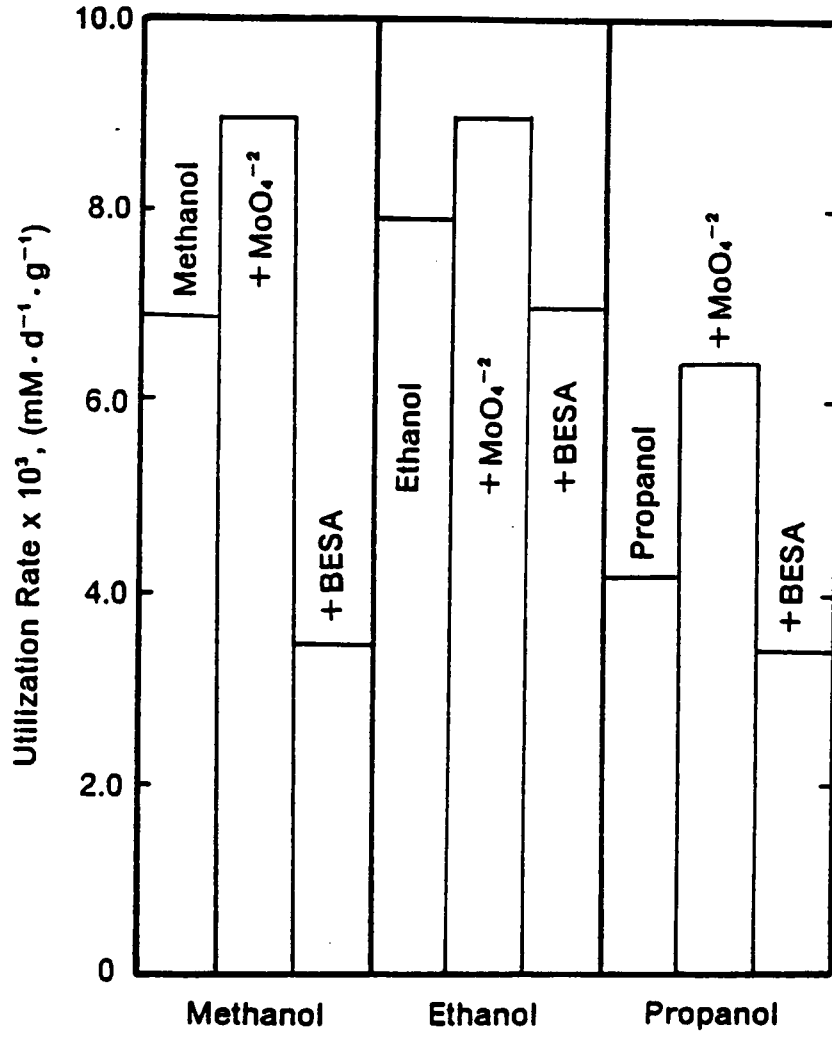

Figure 22. Biodegradation rates of methanol, ethanol and propanol in Blacksburg soil (site 1, 15 feet) with and without molybdate or BESA.

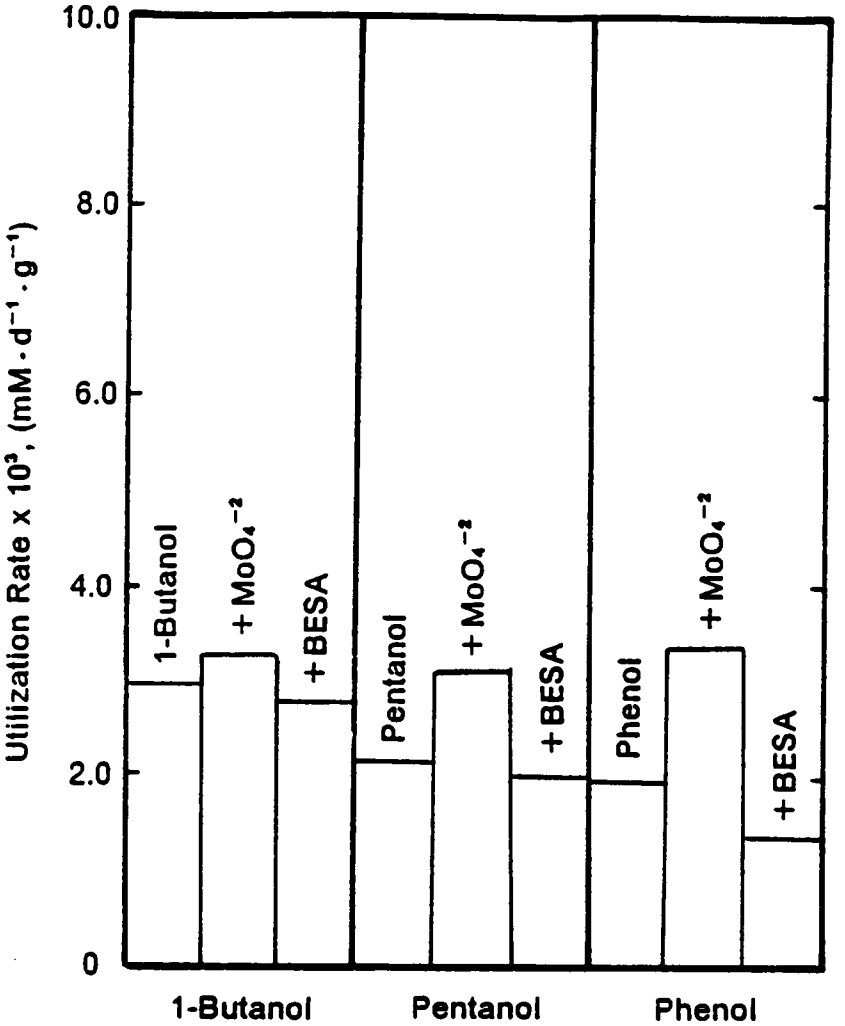

Figure 23. Biodegradation rates of 1-butanol, pentanol and phenol in Blacksburg soil (site 1, 15 feet) with and without molybdate or BESA.

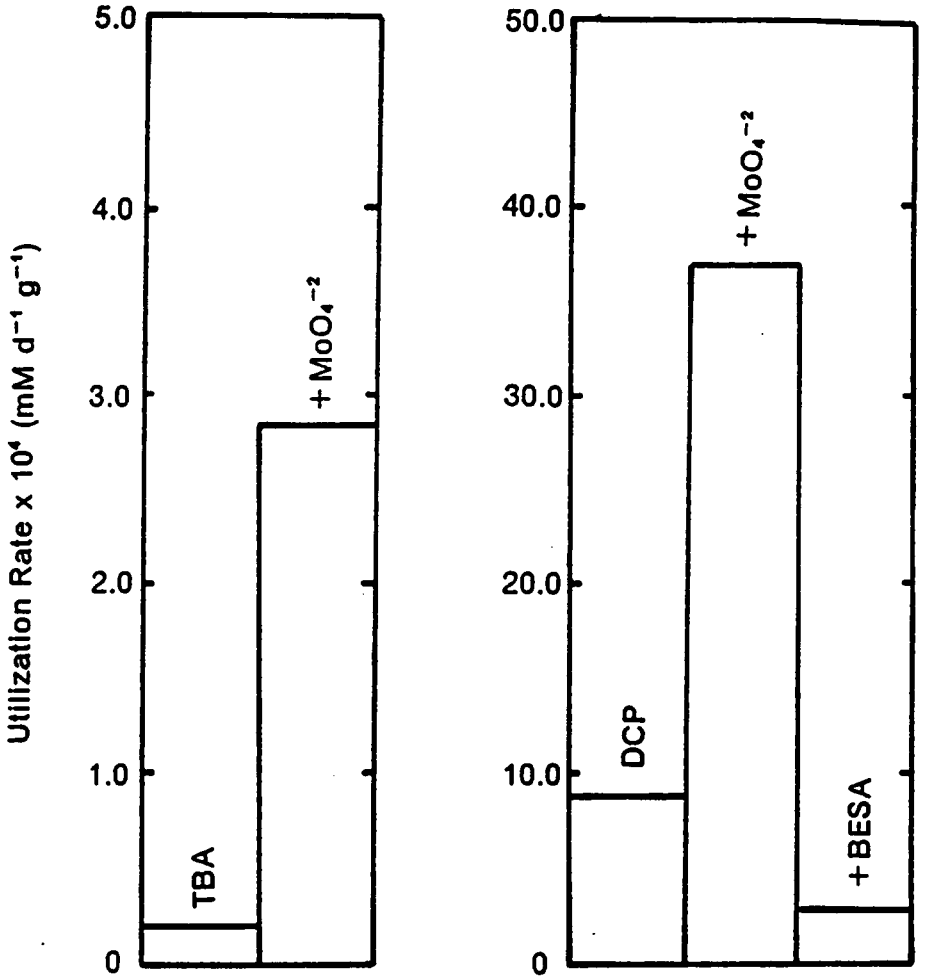

Figure 24. Biodegradation rates of TBA and DCP in Blacksburg soil (site 1, 15 feet) with and without molybdate or BESA.

DCP 70%. The only explanation which can be offered as to why methanol was affected by BESA much more than the other alcohols is that methanol can be converted directly to methane and carbon dioxide by methanogenic bacteria, whereas the other alcohols require an acetogenic step during methanogenic conversion.

The presence of molydate or BESA in microcosms containing Newport News soil did not have the same effect as in the Blacksburg soil. For the alcohols, there was no discernable change in the degradation rate in the presence of either molybdate or BESA except for methanol. There was a 48% decrease in the utilization rate in the presence of BESA. Phenol was affected by BESA slightly and DCP less so, but neither was influenced by molybdate. Figure 25 compares the degradation of Newport News microcosms containing only TBA and TBA plus molybdate. Figures 26 and 27 shows the degradation of methanol and phenol, respectively in the presence of molybdate and BESA in the Newport News soil. Similar plots for the remaining substrates are contained in Appendix B. Figures 28 through 30 show the degradation rates of all the test substrates in the presence of either molybdate or BESA for the Newport News soil.

The results of these experiments suggest that inhibition of sulfate reduction with molybdate increased the utilization rate of all the test compounds in the Blacksburg soil. In particular, TBA and DCP increased by over an order of magnitude. On the other hand, inhibition of methanogenesis with BESA decreased the biodegradation rates. These results are consistent with other studies. In a series of experiments by Suflita and his collegues (1985 and 1986), chloroaromatics were degraded in soil with a distinct presence of methane. These compounds, however, persisted in an actively sulfate reducing soil or when sulfate was added to the methanogenic soil. In this study, 16 mg/l DCP was 50% degraded in about 13 days, while 14 mg/l DCP plus molybdate was 50% degraded in 7 days in the Blacksburg soil. With BESA added, 12.5 mg/l DCP was biodegraded to 50% of the initial concentration in excess of 20 days. This is shown in Figure 31. This particular method of expressing the biodegradation of DCP was chosen since this compound was not monitored to below the detection limit. The results obtained in this study using the Blacksburg soil suggest that the interrelationship between sulfate reduction and

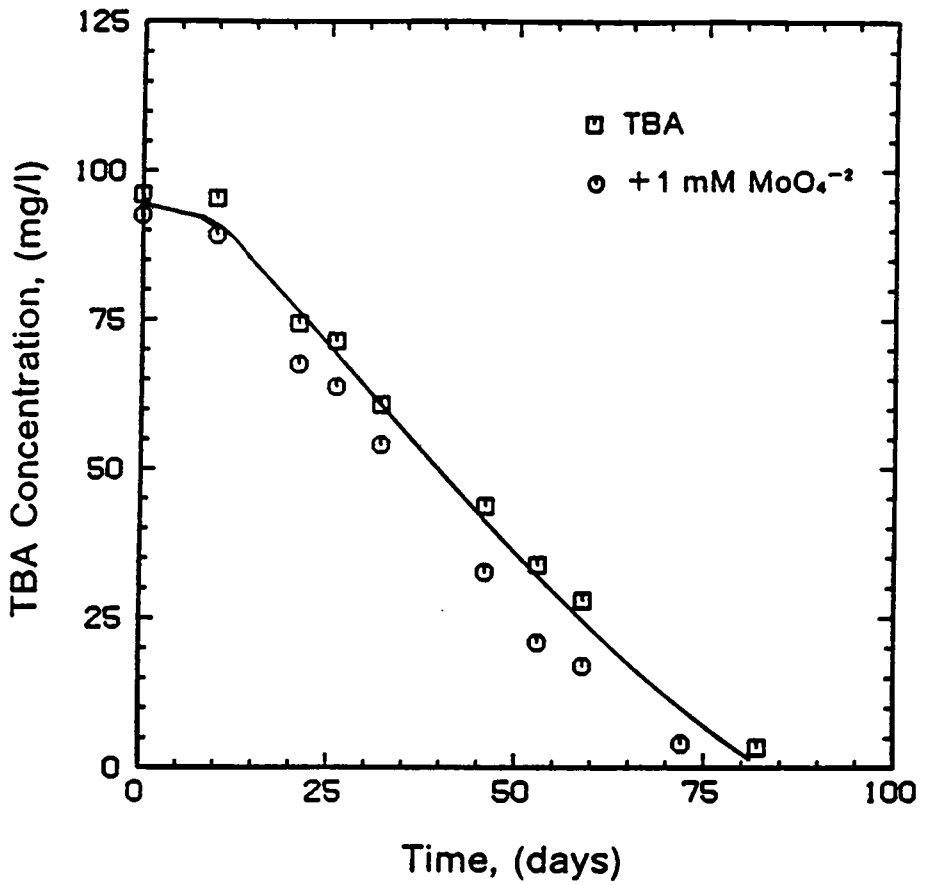

Figure 25. TBA biodegradation in Newport News soil with and without molybdate.

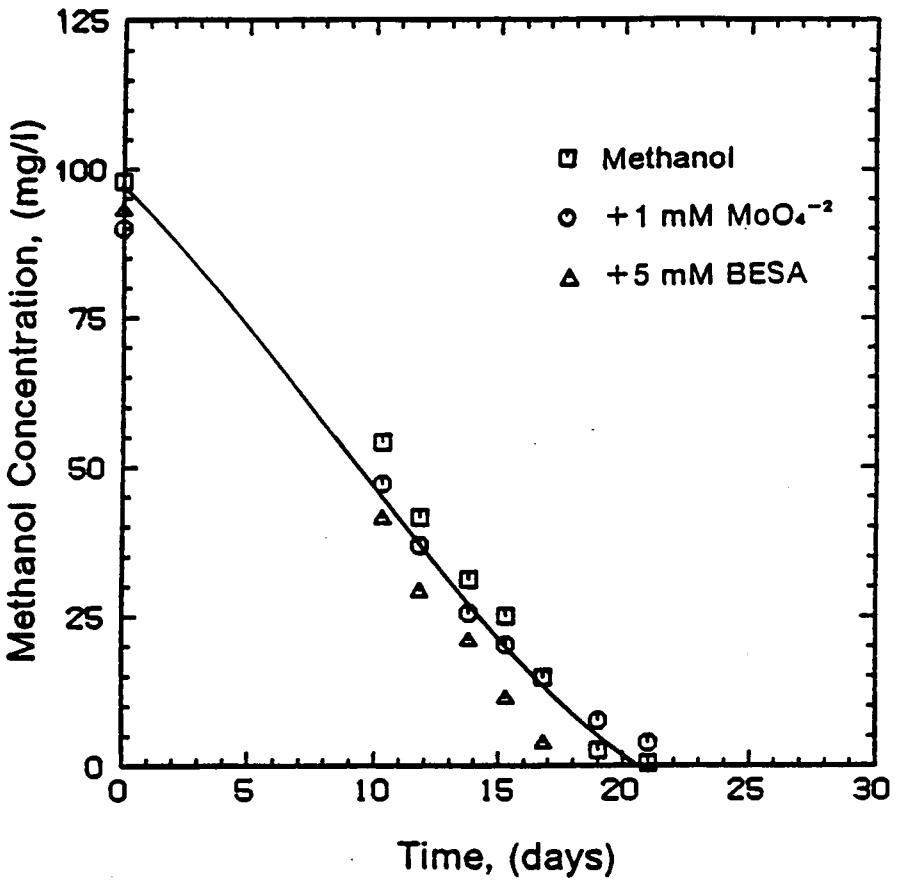

Figure 26. Methanol biodegradation in Newport News soil with and without molybdate and BESA.

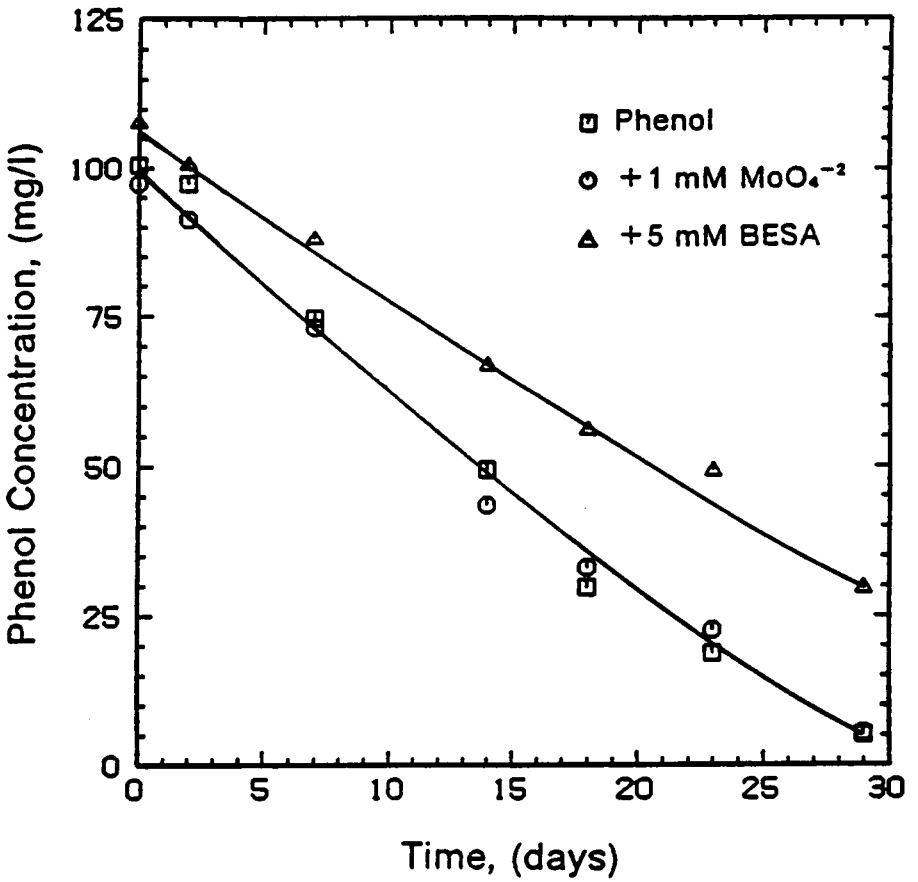

Figure 27. Phenol biodegradation in Newport News soil with and without molybdate and BESA.

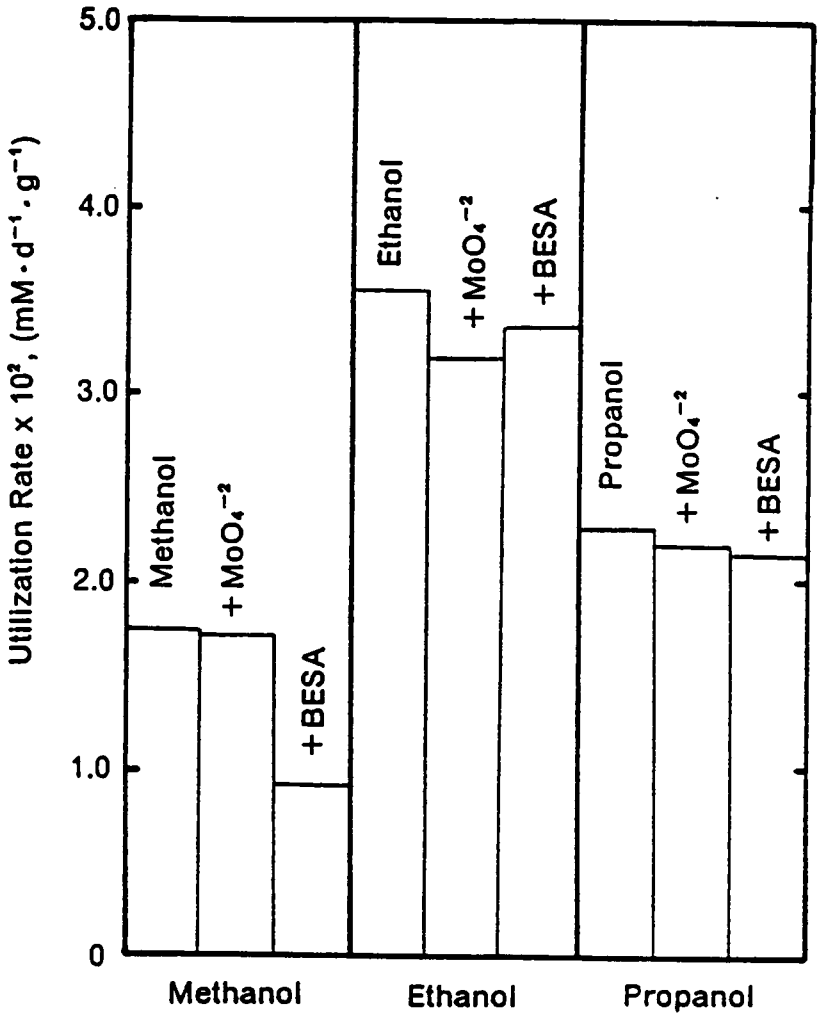

Figure 28. Biodegradation rates of methanol, ethanol and propanol in Newport News soil with and without molybdate or BESA.

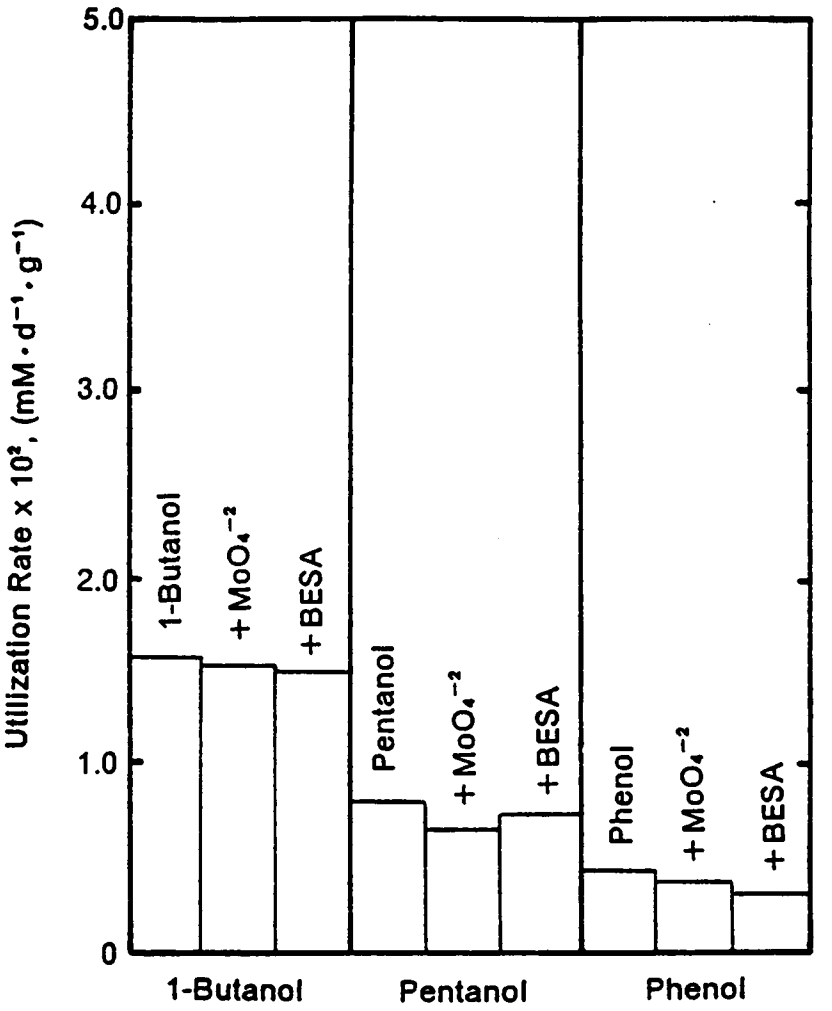

Figure 29. Biodegradation rates of 1-butanol, pentanol and phenol in Newport News soil with and without molybdate or BESA.

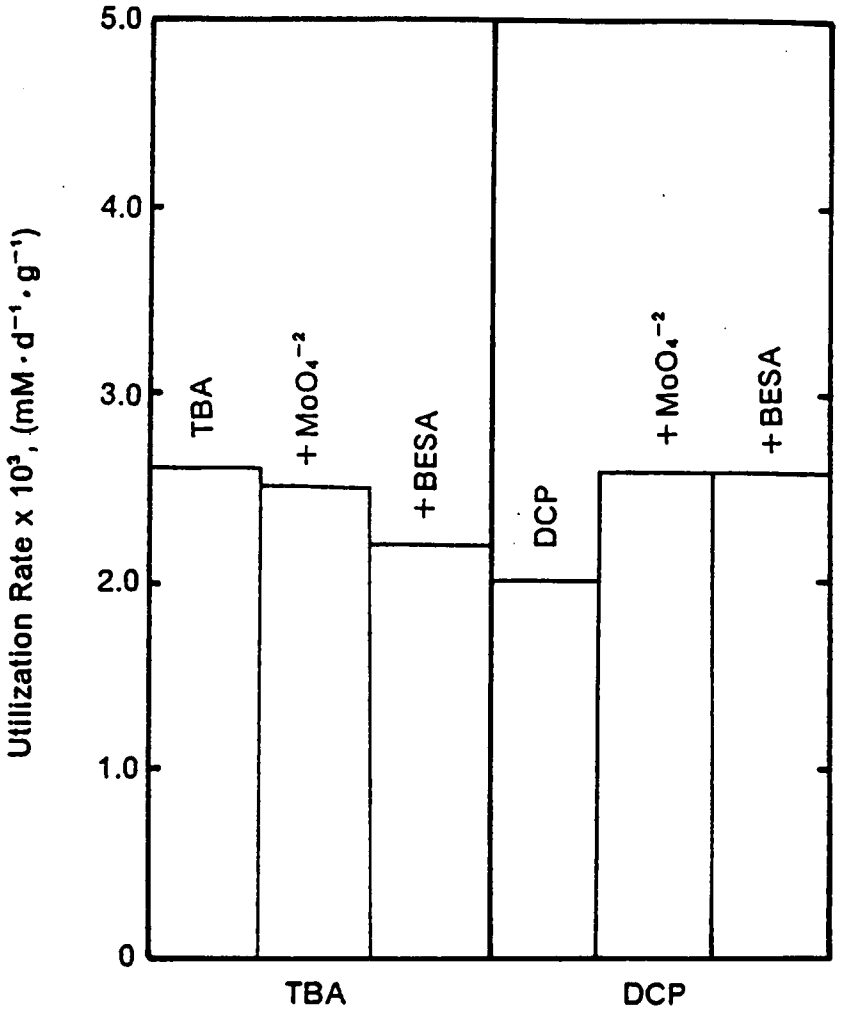

Figure 30. **Biodegradation rates of TBA and DCP in Newport News soil with and without molybdate or BESA.**

methanogenesis not only affects the absolute degradation of certain chemicals as described by Suflita, but the kinetics of degradation for a wide range of compounds. In addition, the Blacksburg soil would not be classified as exclusively sulfate reducing or exclusively methanogenic based on biodegradation since addition of either of the inhibitors did not completely stop the biological response as was observed by Sulflita in his studies.

Though the effect of molybdate in stimulating biodegradation was consistent with all of the test compounds in Blacksburg soil, the magnitude of the response was significantly greater for DCP and TBA than for methanol, ethanol, propanol, 1-butanol, pentanol and phenol. There was no direct evidence in this study which would account for this difference, however, an explanation based on the literature can be offered. As stated previously, numerous studies have shown that in hydrogen limiting environments, sulfate reducing bacteria can outcompete methanogenic bacteria for available hydrogen. In addition, since both sulfate reduction and carbon dioxide reduction require hydrogen, it is reasonable to speculate that molybdate acts to alter the hydrogen flow from sulfate reducers to methanogens. The question which must be answered, therefore, is why does a shift in hydrogen flow affect DCP and TBA degradation rates much more than the rates for the remaining test compounds? The answer to this question may be related to the role of hydrogen in the degradation pathways for each of these compounds. As described previously, the methanogenic conversion of complex organics requires a source of hydrogen as a repository of electrons. DCP, on the other hand, is degraded via reductive dehalogenation in which chlorine is replaced by hydrogen (Boyd, 1983 and; Boyd and Shelton, 1984). In this case, hydrogen is used directly as a structural component. A degradation pathway for TBA has not been reported. TBA, however, is structurally similar to trimethylamine. Hippe, et al. (1979) reported that trimethylamine was degraded in a culture of *Methanosarcina barkeri* producing dimethylamine and methylamine as intermediates. If TBA responded similarly, its degradation would also involve a structural substitution by hydrogen. It may be reasonable, therefore, to propose that inhibition of sulfate reduction by molybdate in the Blacksburg soil enhanced degradation more for those compounds which require hydrogen in a

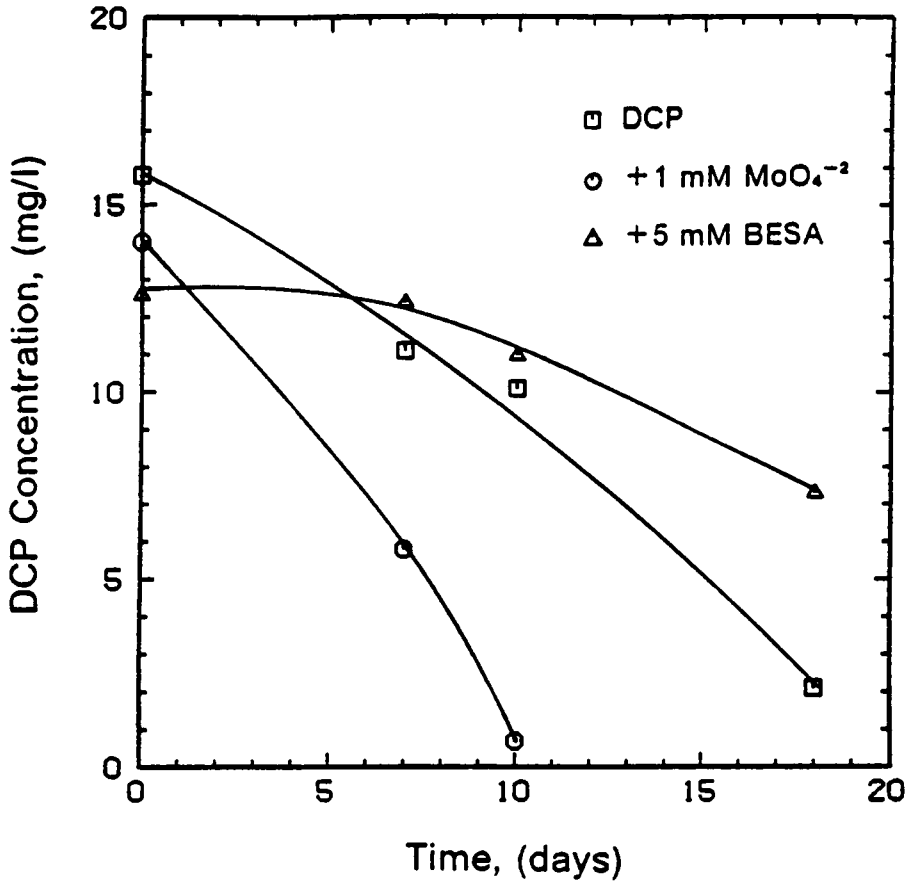

Figure 31. DCP biodegradation in Blacksburg soil (site 1, 15 feet) with and without molybdate and BESA.

structural position than for those compounds which use hydrogen as a reducing equivalent. Obviously, much more research is needed to support this hypothesis.

In contrast to the results observed in the Blacksburg soil, molybdate and BESA did not affect degradation rates in the Newport News soil. The Newport News soil was more biologically active than Blacksburg soil as evidenced by the black FeS which was visibly evident and a gas chromatographic peak corresponding to methane which was detected. This indicated that both sulfate reduction and methanogenesis was occurring. When molybdate was present, no black color was present and when BESA was added, there was no methane peak. The degradation rates, however, did not vary in the presence of either of the inhibitors. The Newport News site was located next to and below the water level of a resevoir. It is likely that this water was a significant source of organic carbon and nutrients to the subsurface microflora resulting in a much more active population than was found in the Blacksburg soil. Consequently, the competition between sulfate reducers and methanogens was not in evidence.

There are indications in previous work with methanol and TBA that additions of sulfate can affect biodegradation rates. In a study on the use of alternative electron acceptors in subsurface biodegradation of methanol and TBA, Mulheren (1985) observed that sulfate slightly decreased methanol utilization rate in Dumfries, Virginia soil. In non-amended soil 90 mg/l methanol was 50% degraded in 33 days, whereas, a similar concentration of methanol plus 50 mg/l SO_4^{-2} was degraded by 50% in about 50 days. By day 45 of the analysis, the degradation rate in non-amended methanol microcosms was 1.5 mg/l/day, but was 0.9 mg/l/day in microcosms dosed with 50 mg/l SO_4^{-2}. This observation, however, was not consistent in all experiments possibly because the soil had been stored for a considerable amount of time.

4.6. Nitrate Addition

Nitrate was added to microcosms containing each of the test substrates to determine the potential for nitrate reduction in each of the soils. In the Blacksburg soil, nitrate did not affect the degradation pattern in any of the test compounds. Figures 32 through 34 show the biodegradation of TBA, methanol and phenol, respectively, with 0.8 mM added nitrate. Figures 60 through 64 in Appendix A show the degradation of the remaining test compounds in the presence of nitrate. Mulheren (1985) observed nitrate reduction in Dumfries, Virginia microcosms comtaining methanol plus nitrate. In some instances, however, nitrite accumulated inhibiting further degradation. This was attributed to low pH conditions which is known to aggravate nitrite toxicity. Given that the pH of the Blacksburg soil was 4.5, nitrite toxicity was suspected to occur in these microcosms. Nitrate addition, however, had no effect on degradation possibly indicating the absence of an active nitrate reducing population in the Blacksburg soil at a depth of 15 feet. Figures 35 and 36 show the degradation rates for all the test compounds with and without nitrate.

In contrast to the Blacksburg soil, nitrate stimulated degradation in Newport News soil. The addition of 1.6 mM NO_3^- to microcosms decreased the time necessary to biodegrade approximately 95 mg/l TBA from 85 days to 50 days (Figure 37). Likewise, 95 mg/l methanol (Figure 38) degraded in 21 days in the presence of 1.6 mM NO_3^-, but required about 27 days in non-amended microcosms. As Figure 39 indicates, however, phenol was not affected by the addition of nitrate. Figures 74 through 78 in Appendix B shows the degradation of the remaining compounds with added nitrate in Newport News soil. Figures 40 and 41 show the degradation rates for the test compounds with and without nitrate in the Newport News soil. The addition of nitrate increased the methanol degradation rate by 26%, ethanol 51%, propanol 24%, 1-butanol 77%, pentanol 60%, phenol 4%, DCP 35% and TBA 62%.

After the initial inhibition and nitrate addition studies, a second set of experiments were designed using the Blacksburg soil to answer the following questions: 1. Was the stimulation of substrate utilization by molybdate due to incorporation of the trace element molybdenum? 2. Was

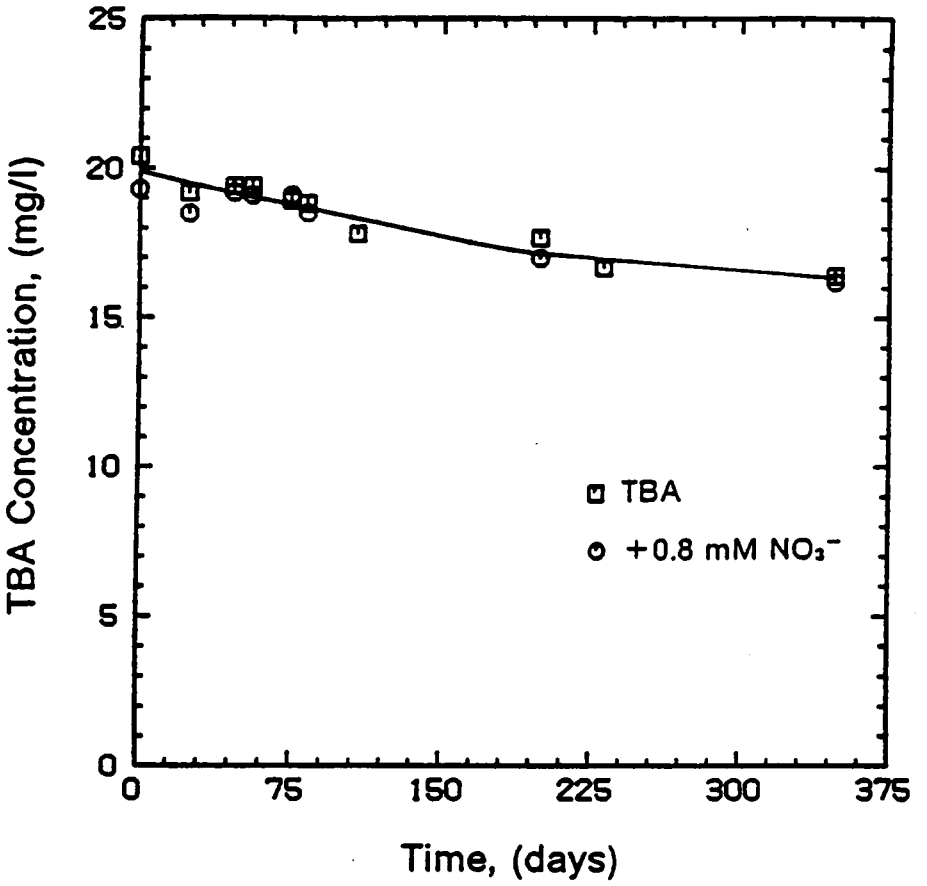

Figure 32. TBA biodegradation in the presence of nitrate in Blacksburg soil (site 1, 15 feet).

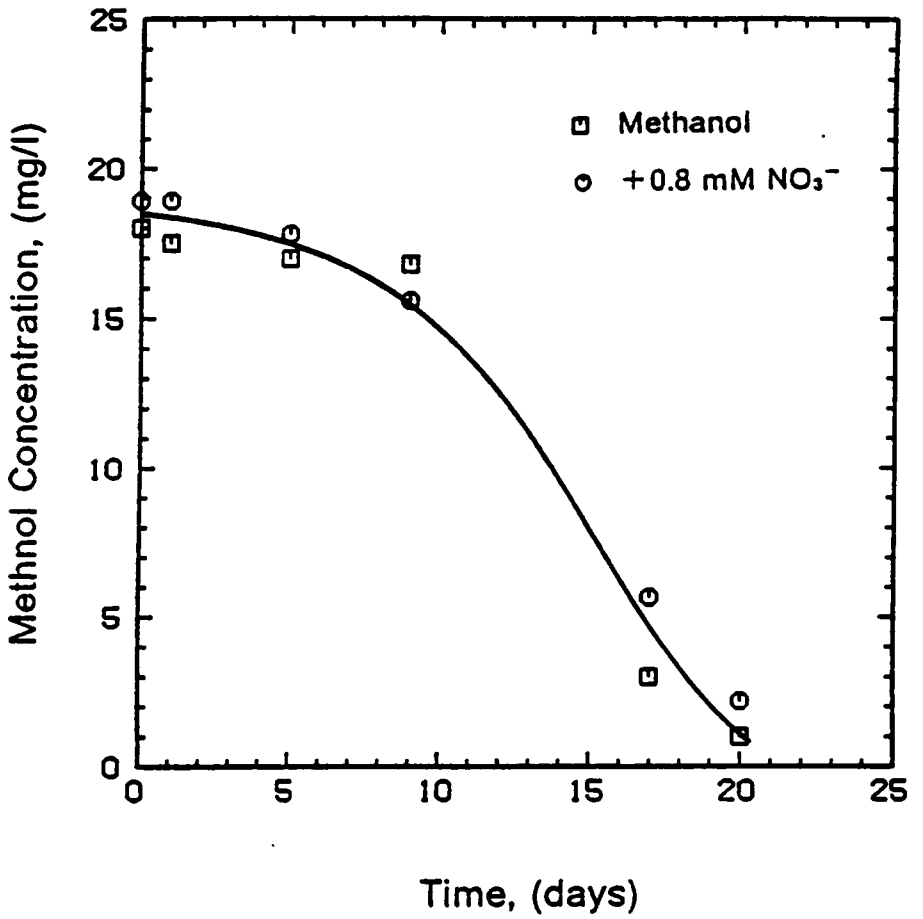

Figure 33. Methanol biodegradation in the presence of nitrate in Blacksburg soil (site 1, 15 feet).

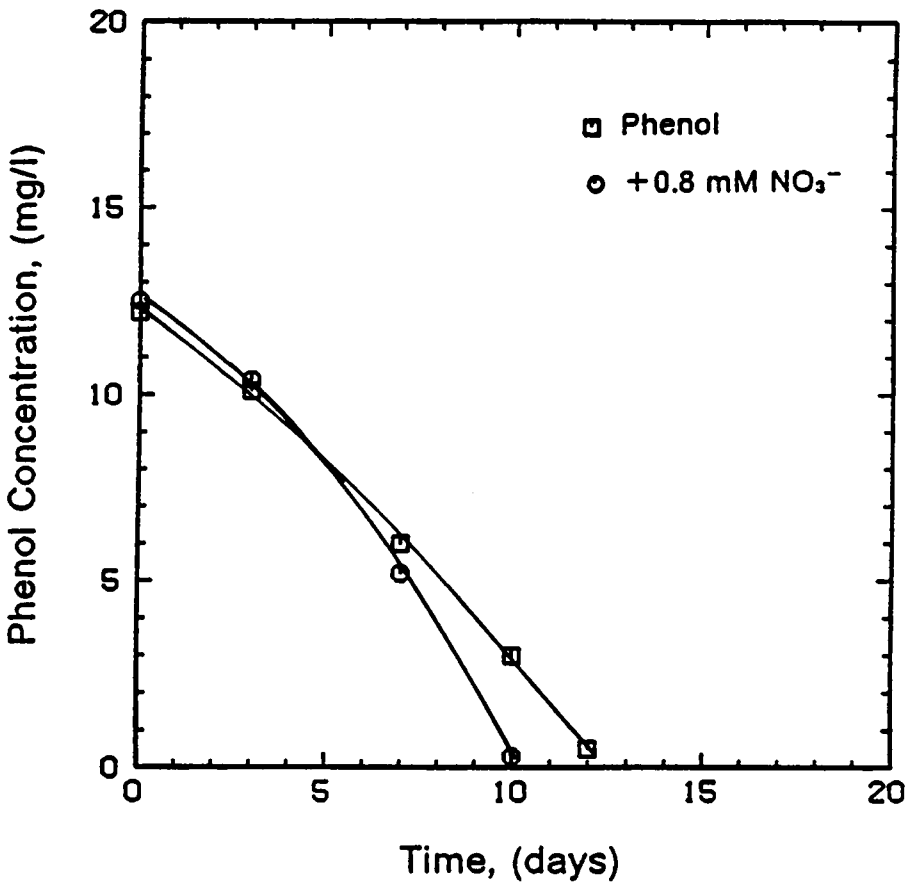

Figure 34. Phenol biodegradation in the presence of nitrate in Blacksburg soil (site 1, 15 feet).

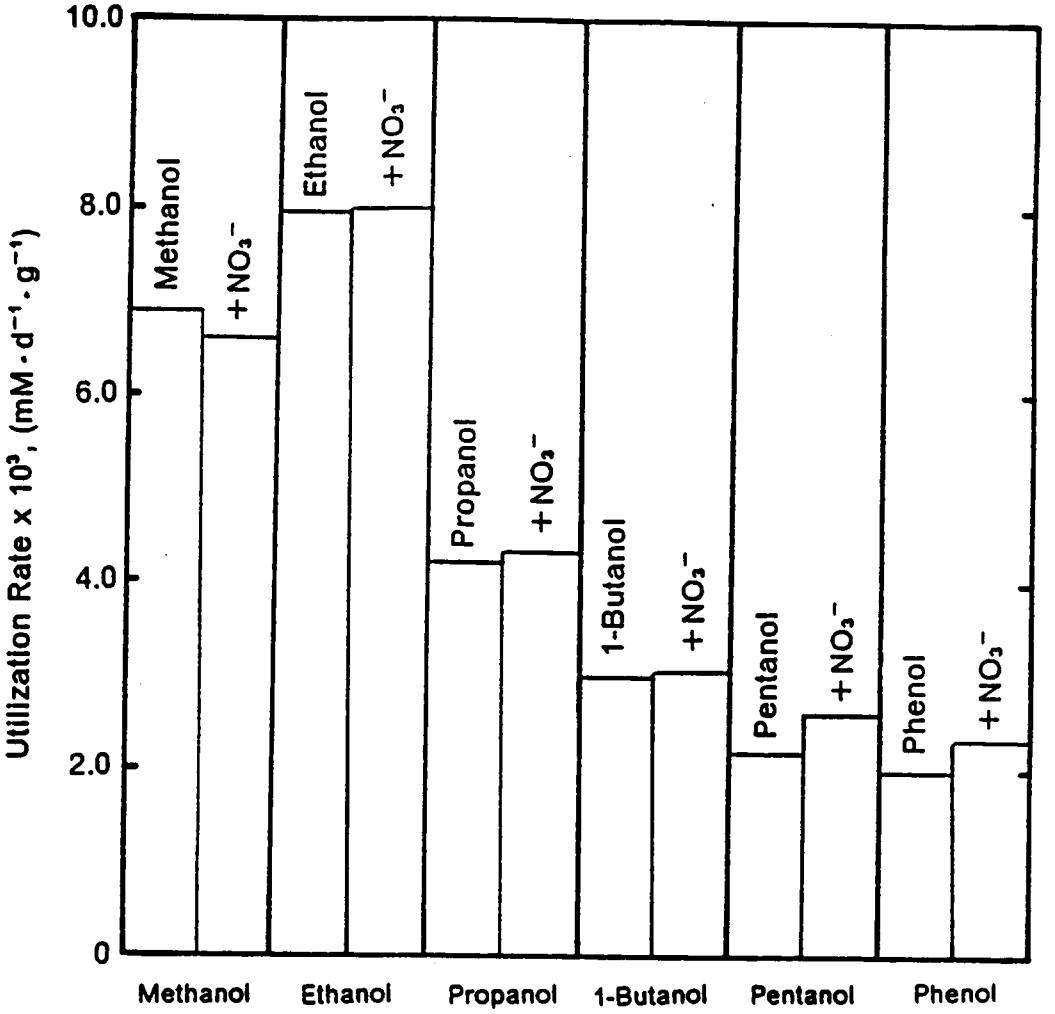

Figure 35. Biodegradation rates of methanol, ethanol, propanol, 1-butanol, pentanol and phenol in Blacksburg soil (site 1, 15 feet) with and without 0.8 mM nitrate.

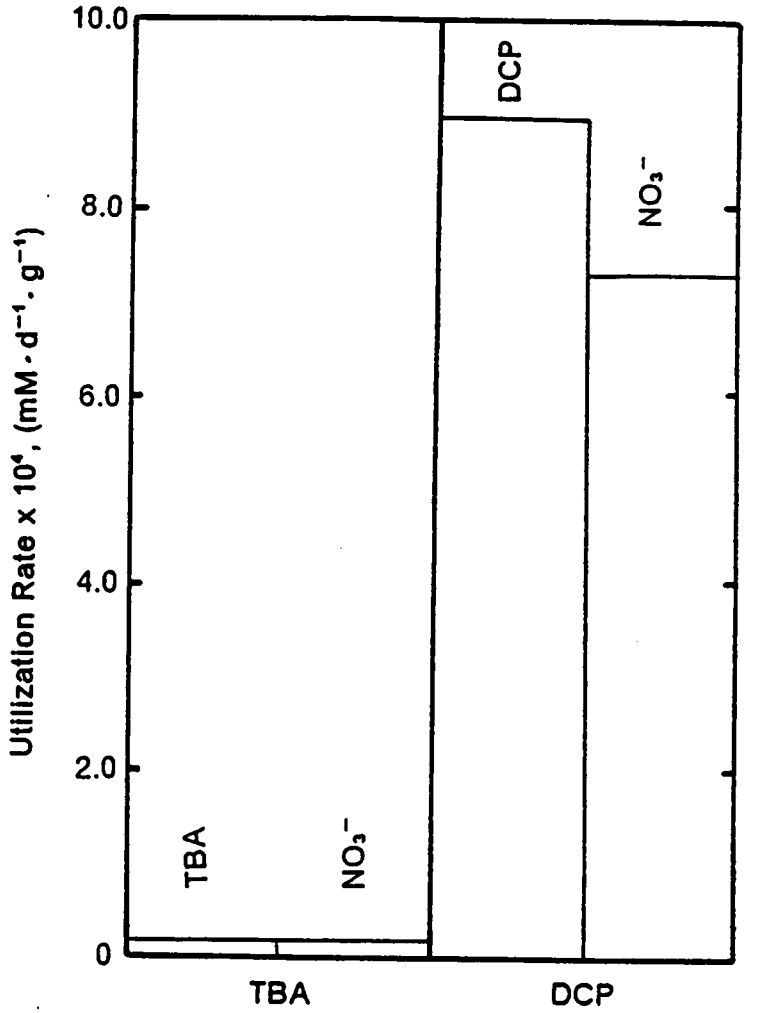

Figure 36. Biodegradation rates of TBA and DCP in Blacksburg soil (site 1, 15 feet) with and without 0.8 mM nitrate.

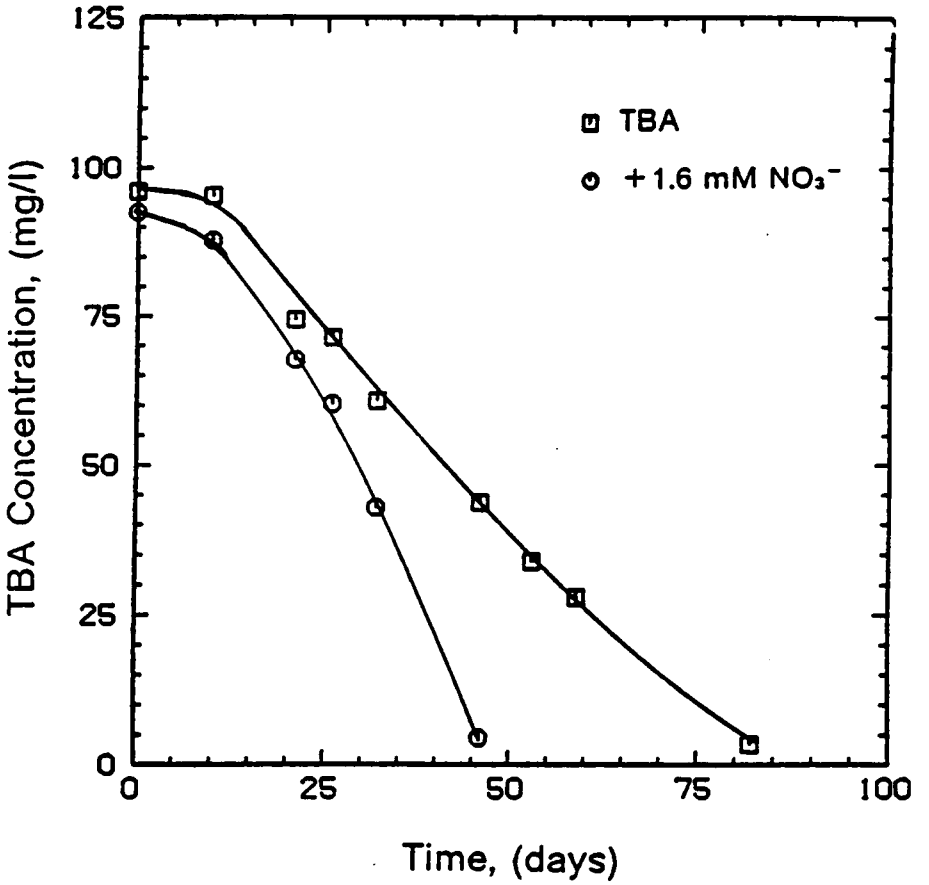

Figure 37. TBA biodegradation in the presence of nitrate in Newport News soil.

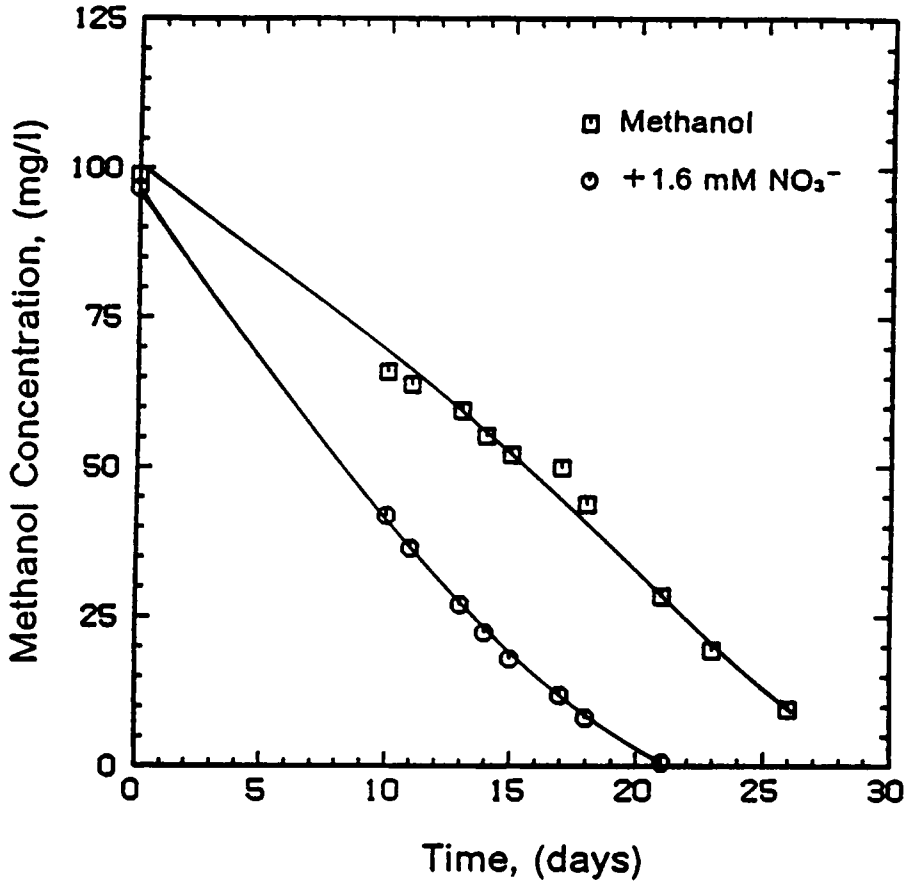

Figure 38. Methanol biodegradation in the presence of nitrate in Newport News soil.

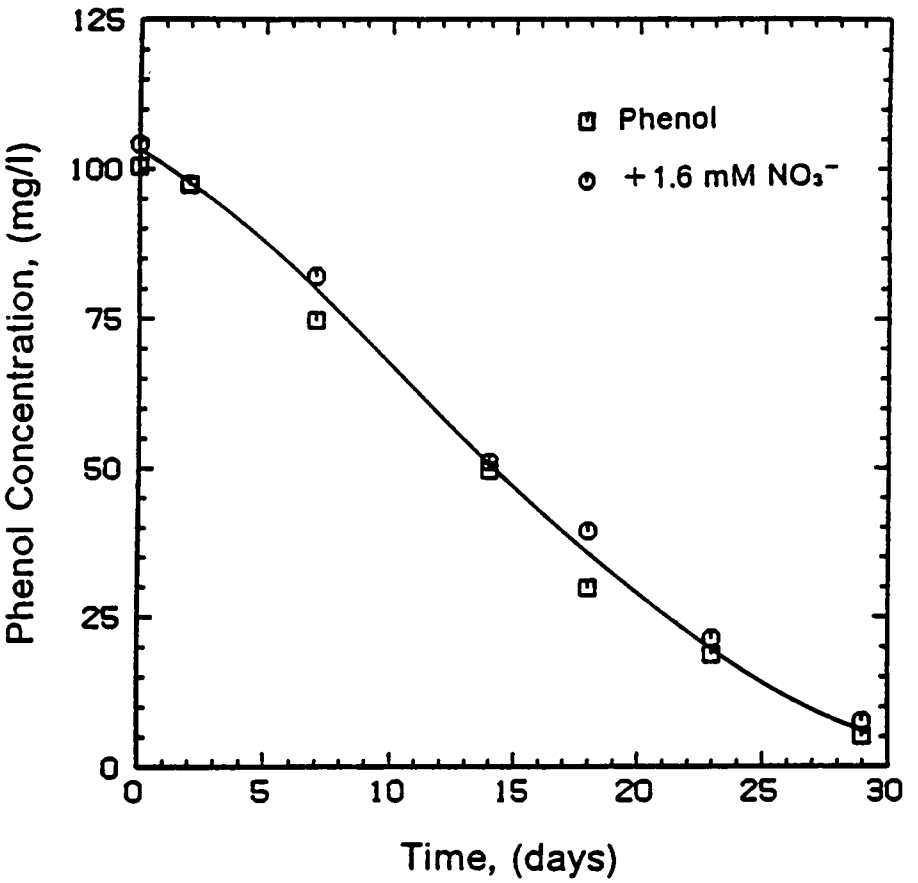

Figure 39. Phenol biodegradation in the presence of nitrate in Newport News soil.

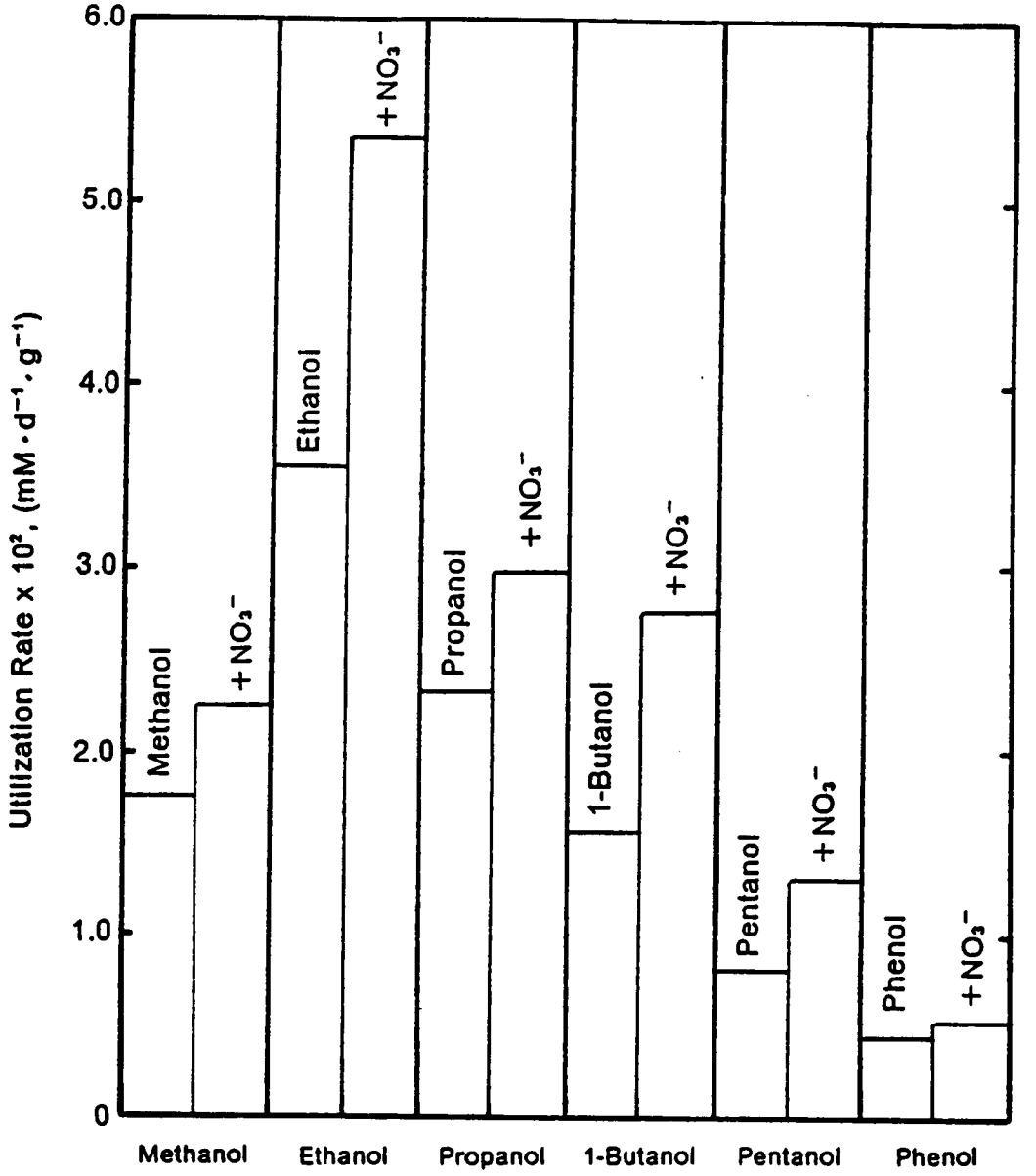

Figure 40. **Biodegradation rates of methanol, ethanol, propanol, 1-butanol, pentanol and phenol in Newport News soil with and without 1.6 mM nitrate.**

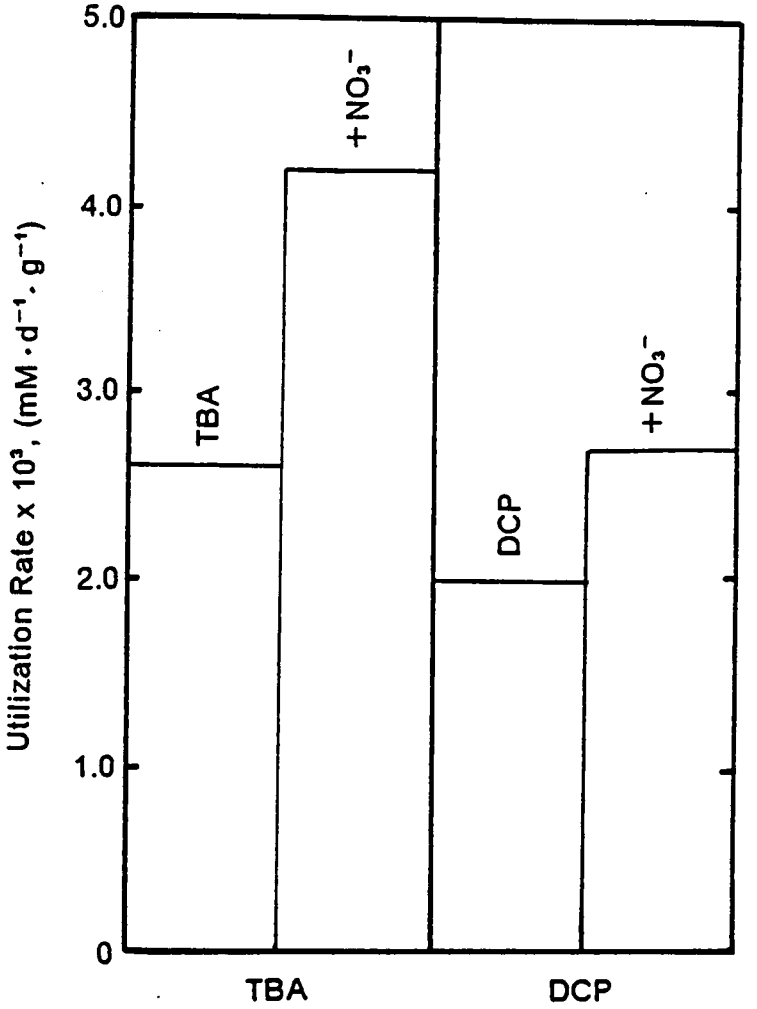

Figure 41. Biodegradation rates of TBA and DCP in Newport News soil with and without 1.6 mM nitrate.

molybdate involved in the stimulation of nitrate reduction since molybdenum is a requirement in the nitrate reductase enzyme? 3. Did TBA degradation involve the accumulation of an intermediate which might feedback and inhibit further degradation? 4. Did molybdate alleviate a feedback inhibition by creating conditions favorable for the degradation of the intermediate? 5. What were the gaseous end products of degradation in the presence of molybdate and BESA? To answer these questions, additional microcosms were constructed using Blacksburg soil obtained from a second site located about 500 yards from the first site. This soil was taken from a depth of about four feet.

The results of the second study indicated that the soil from Blacksburg site 2 was not similar to site 1 soil in its response to molybdate or nitrate. In the Blacksburg site 2 soil, molybdate did not stimulate the degradation of any of the test organics. Nitrate plus molybdate, however, did increase the degradation rates of the test compounds. Since molybdate alone did not affect degradation, stimulation in microcosms containing nitrate plus molybdate must be attributed to the presence of nitrate. The addition of molybdenum initially inhibited substrate utilization for all the test compounds. If molybdenum acted in the same fashion as molybdate, no effect in the degradation rate would have been observed in the soil from the second site. It can be assumed, therefore, that the stimulatory effect of molybdate in microcosms containing Blacksburg site 1 soil was not due to the incorporation of the molybdenum atom as a trace element. Figures 42 and 43 show these results for methanol and phenol. Similar figures for the remaining test compounds are contained in Appendix A. In addition, no gaseous end products or TBA intermediates were detected. On one hand, these experiments using soil from site 2 were not viewed as a success because the condition of this soil was much different than soil from site 1. In retrospect, this is not surprising because of the differences between the depth from which each soil was sampled. Site 2 soil taken from four feet would be expected to contain a high population of facultative bacteria some of which were capable of reducing nitrate. Site 1 soil which was taken from 15 feet was probably populated by more strictly anaerobic bacteria. On the other hand, however, the results obtained form this soil indicated that significantly different responses can be obtained in two soils which have been col-

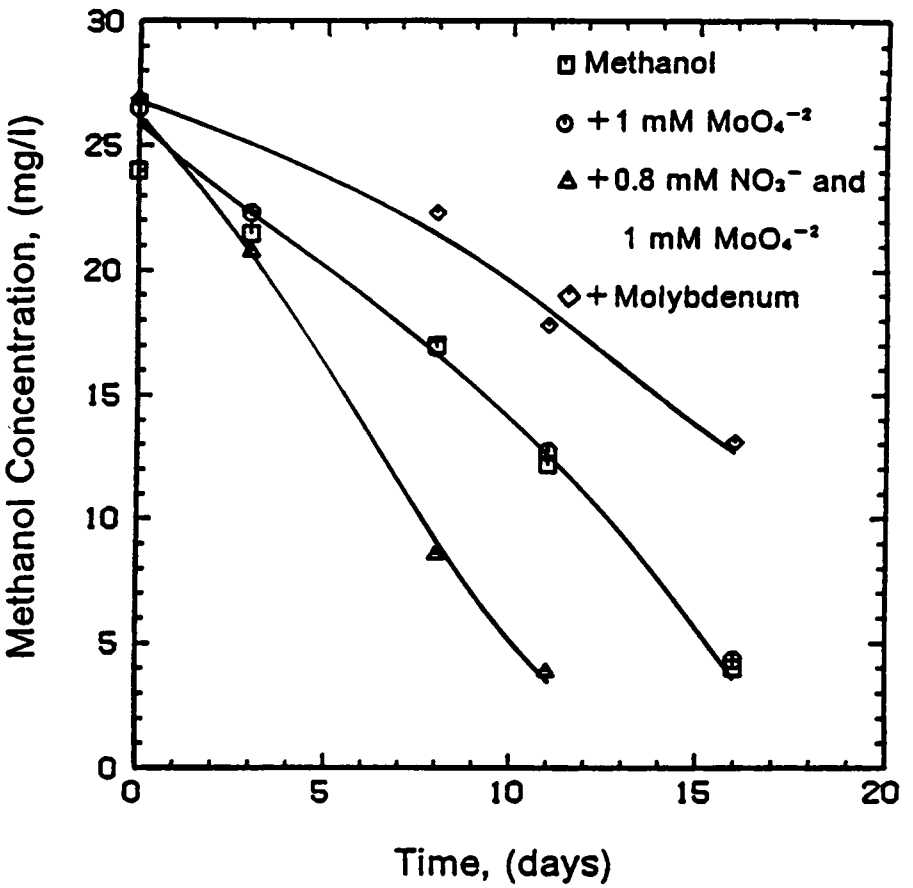

Figure 42. Methanol biodegradation in Blacksburg soil (site 2, 4 feet) with and without molybdate, nitrate plus molybdate and molybdenum.

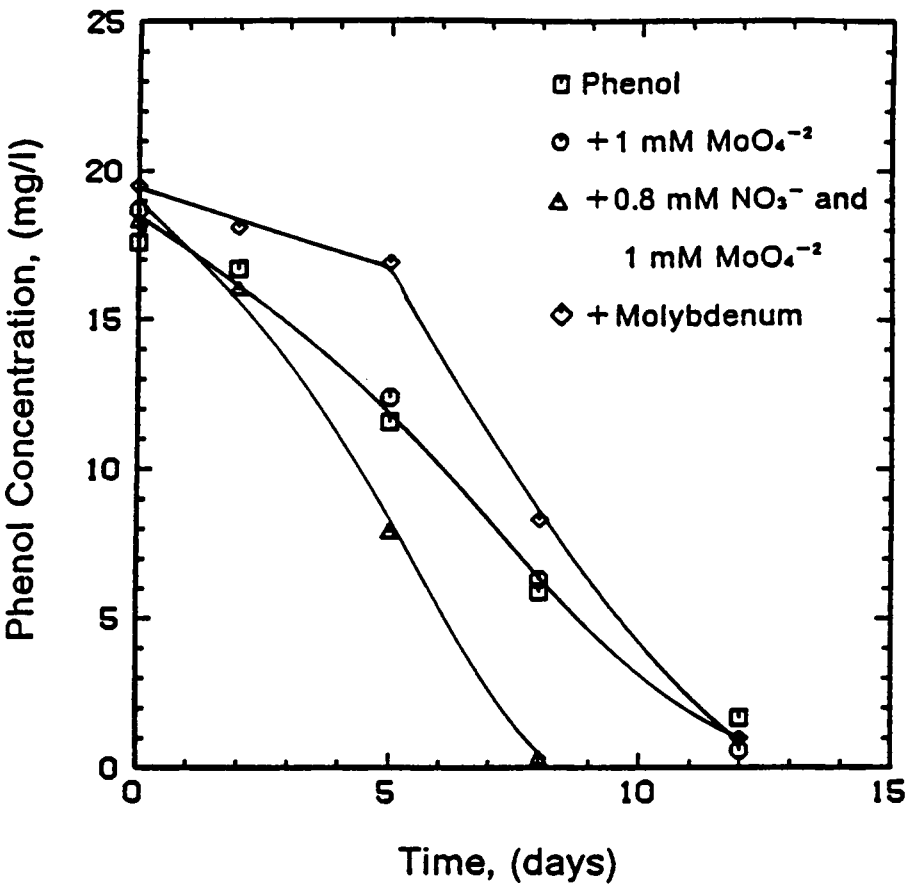

Figure 43. Phenol biodegradation in Blacksburg soil (site 2, 4 feet) with and without molybdate, nitrate plus molybdate and molybdenum.

lected in the same general area. This reflects the importance of the soil microbial ecology as opposed to the soil physical characteristics in controlling biodegradation.

4.7. Kinetics

This study was initiated in large part to examine and explain the kinetics of TBA degradation in the subsurface. As discussed earlier, Goldsmith (1985) and White (1986) observed that TBA degradation was first order with respect to initial concentration in soil obtained from Wayland, New York and Dumfries, Virginia. A similar evaluation was performed using data obtained in this study with the Blacksburg (site 1) and Newport News soils. Since this study used batch microcosms, average substrate utilization rates as determined by the method shown in Figure 8 and normalized for the amount of soil in each microcosm were used to approximate the specific substrate utilization rate. As shown in Figure 44, a logarithmic plot of TBA utilization rate versus initial concentration in the Blacksburg soil yielded a similar relationship as was observed in the Wayland and Dumfries soils. In general, a 10-fold increase in initial concentration resulted in a 10-fold increase in the utilization rate over the range of concentrations used in this study. For the Newport News soil, however, the degradation rate was approximately constant when the initial concentration was greater than 10 mg/l. At an initial concentration of about 1 mg/l, the degradation rate was about 10 times less than the rate at 10 mg/l. This relationship is shown in Figure 45.

The biological response to a single substrate is often characterized using the Michaelis-Menten equation describing enzyme kinetics.

$$r = \frac{R(S)}{K_m + (S)} \tag{4.7a}$$

where:

r = reaction rate, time^{-1}

R = maximum rate of product formation, time^{-1}

S = substrate concentration, mass volume^{-1}

K_m = saturation constant equal to substrate concentration when $r = R/2$, mass volume^{-1}

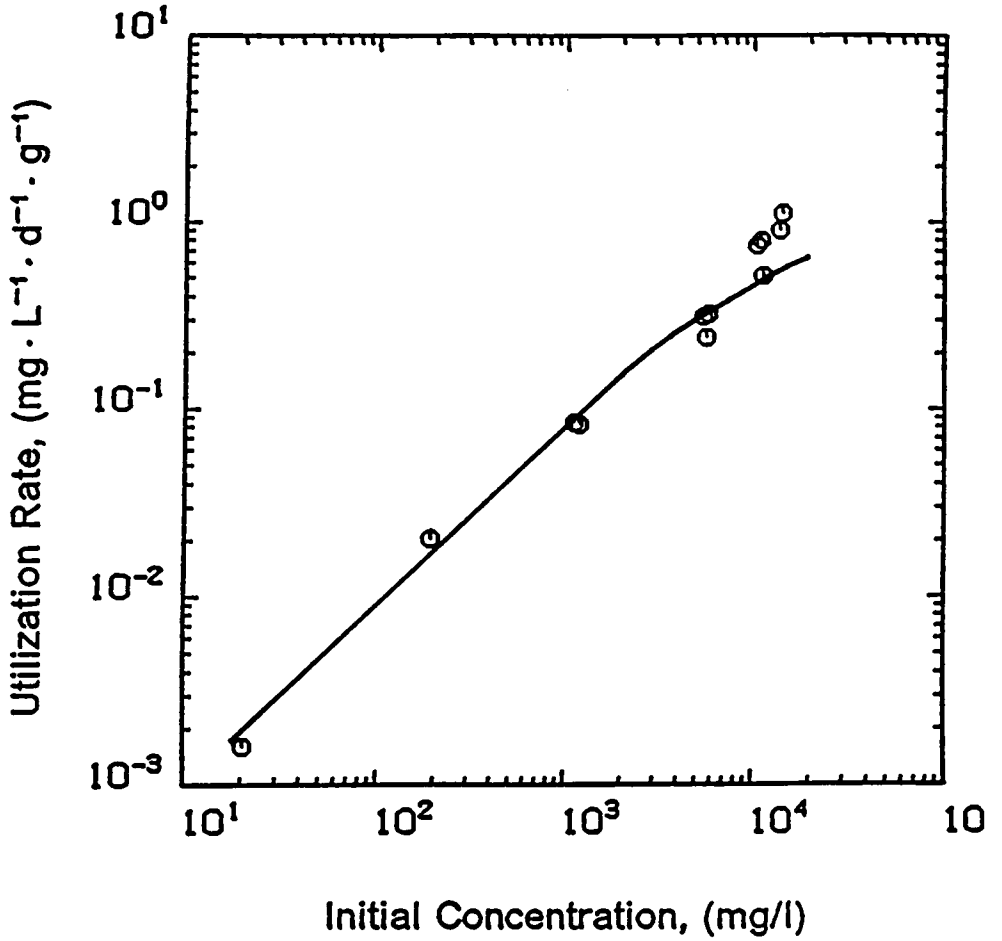

Figure 44. Determination of TBA utilization response in relation to initial concentration for Blacksburg soil (site 1, 15 feet).

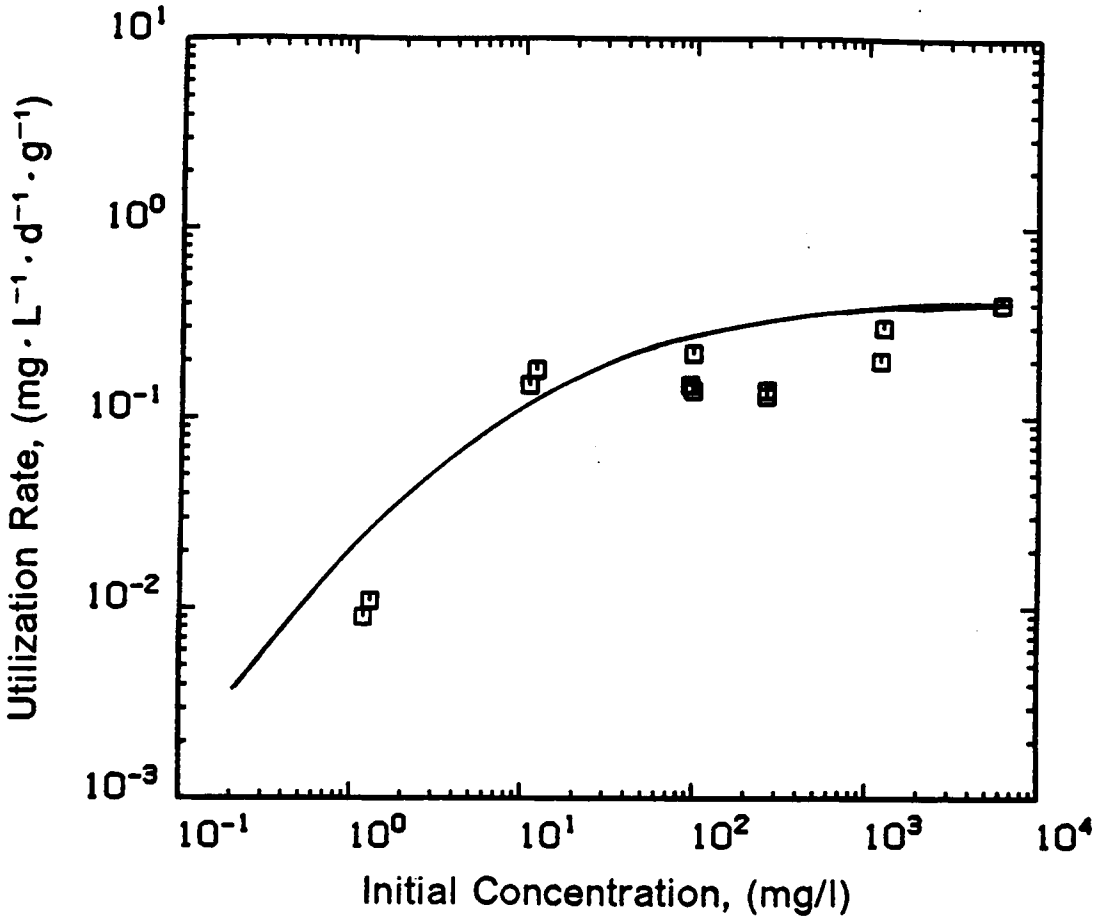

Figure 45. Determination of TBA utilization response in relation to initial concentration for Newport News soil.

This relationship was derived based on a model involving the reversible reaction between the substrate and an enzyme forming an enzyme/substrate complex. This complex the irreversibly decomposes into a product and free enzyme. Steady state conditions result when the enzyme/substrate complex concentration is constant. The value of K_m is reflected in the individual rate constants for these reactions. The maximum rate of product formation (R) occurs when all the enzyme is complexed. This requires an excess of substrate (Benefield and Randall, 1980).

Monod modified the Michaelis-Menten equation to describe the growth rate of a bacterial population in the presence of a growth limiting substrate.

$$\mu = \frac{\mu_m(S)}{K_s + S} \tag{4.7b}$$

where:

μ = specific growth rate, time^{-1}

μ_m = maximum specific growth rate, time^{-1}

S = growth limiting substrate concentration, mass volume^{-1}

K_s = saturation constant, mass volume^{-1}

This equation assumes that growth rate is only a function of the organism and substrate concentrations. Other environmental factors, however, can influence growth. The Monod equation is commonly used in biological wastewater treatment where the value of the substrate concentration is estimated by the ultimate biochemical oxygen demand (BOD_u), chemical oxygen demand (COD) or total organic carbon (TOC).

Lawrence and McCarty proposed a similar relationship between substrate utilization rate and the biomass concentration.

$$(\frac{dS}{dt})_u(\frac{1}{X}) = \frac{kS}{K_s + S} = q \tag{4.7c}$$

where:

$(\frac{dS}{dt})_u$ = substrate utilization rate, mass volume^{-1} time^{-1}

k = maximum substrate utilization rate, time^{-1}

x = biomass concentration, mass volume^{-1}

q = specific substrate utilization rate, time^{-1}

Figure 46 shows a graphical representation of this relationship. In this case, substrate utilization is only a function of organism and substrate concentration over the entire range of substrate concentrations. No specific biochemical mechanisms are implied from this relationship. Equation 4.7c can be simplified by considering the limiting cases. If the substrate concentration is assumed to be much greater than K_s, the denominator in equation 4.7c can be approximated as just the substrate concentration. This equation can then be reduced to a zero order expression with respect to substrate concentration which is given by:

$$(\frac{dS}{dt})_u(\frac{1}{x}) = k \tag{4.7d}$$

On the other hand, if the substrate concentration is much less than K_s , the denominator can be approximated by K_s. Equation 4.7c then reduces to a first order expression with respect to substrate concentration and is given by:

$$(\frac{dS}{dt})_u(\frac{1}{x}) = \frac{k}{K_s}S \tag{4.7e}$$

This method of characterizing substrate utilization in terms of the limiting cases is referred as the discontinuous model for substrate utilization (Benefield and Randall, 1980).

The kinetic constants k and K_s can be determined by using the Lineweaver-Burk double reciprocal modification of equation 4.7c.

$$\frac{1}{q} = (\frac{K_s}{k})(\frac{1}{S}) + \frac{1}{k} \tag{4.7f}$$

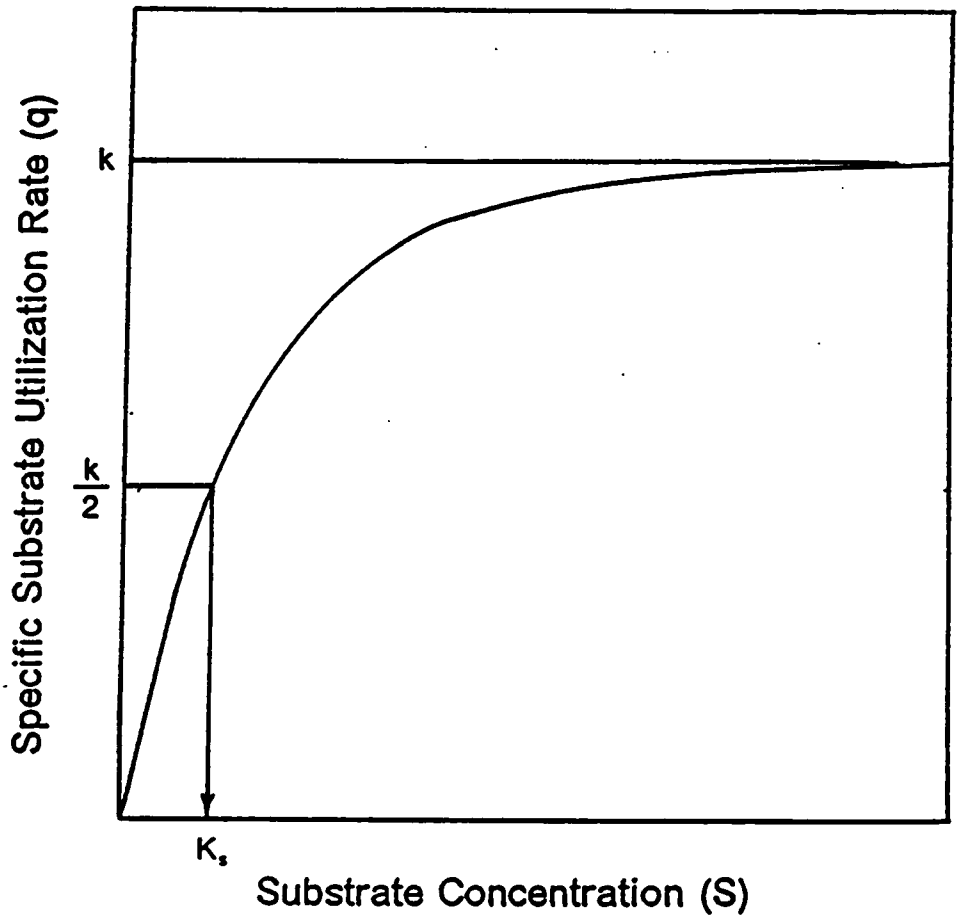

Figure 46. Graphical representation of the Monod equation.

By plotting 1/q versus 1/S, K_s/k can be determined by taking the slope of the resulting line, whereas 1/k is the y-intercept. By using this method for TBA utilization in the Blacksburg soil, K_s was equal to approximately 9600 mg/l and k was equal to 0.83 mg L^{-1} day^{-1} g $soil^{-1}$. These values were used to construct the line on Figure 44. The high value for K_s would indicate that the maximum substrate utilization rate will be achieved at high concentrations and that TBA will persist in the Blacksburg soil at low concentrations. Under most environmental conditions, therefore, TBA concentration will be much less than K_s. The limiting case as shown in equation 4.7e would predict a first order relationship with respect to substrate concentration.

For TBA utilization in the Newport News soil, K_s was equal to 19 mg/l and k was equal to 0.36 mg L^{-1} day^{-1} g $soil^{-1}$. These constants were used to construct the line on Figure 45. The relatively low value of K_s indicates that TBA can be effectively degraded in this soil even at low concentrations. The value of these kinetic constants, however, are somewhat surprising considering the environmental conditions at this site. The dissolved oxygen concentration of the groundwater was less than 1 mg/l. Low values of K_s are typically associated with aerobic respiration, whereas, methanogenic, low temperature conditions usually have higher half saturation constants (Novak, et al., 1974). The dissolved oxygen of the Newport News groundwater measured at the site was 0.5 mg/l. The response of the Newport News soil may be because of its proximity to a reservoir which could supply nutrients during the flow of water through the subsurface.

This procedure was repeated in the Blacksburg soil using phenol as the primary substrate. In this case, K_s was equal to 1.8 mg/l and k was equal to 0.74 mg L^{-1} day^{-1} g $soil^{-1}$. The constants were used to construct the line on Figure 47. In contrast to TBA in the Blacksburg soil, phenol degradation would be rapid at at low initial concentrations.

As shown previously, TBA degradation in Blacksburg soil containing molybdate exhibited a significantly different response than microcosms without molybdate. This is shown in the logarithmic plot of utilization rate versus initial concentration (Figure 48). A reciprocal plot reveals that TBA degradation with molybdate has a K_s equal to 365 mg/l, and k equal to 0.55 mg L^{-1} day^{-1}

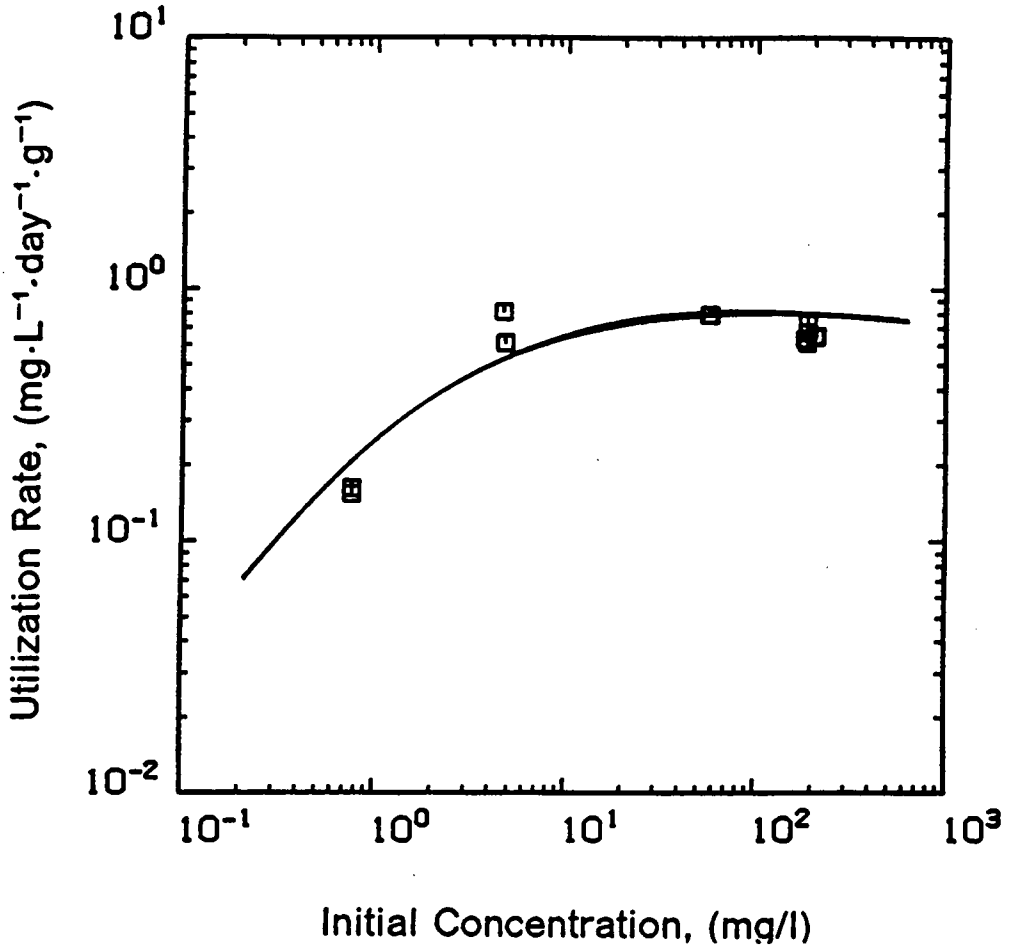

Figure 47. Determination of phenol utilization response in relation to initial concentration for Blacksburg soil (site 1, 15 feet).

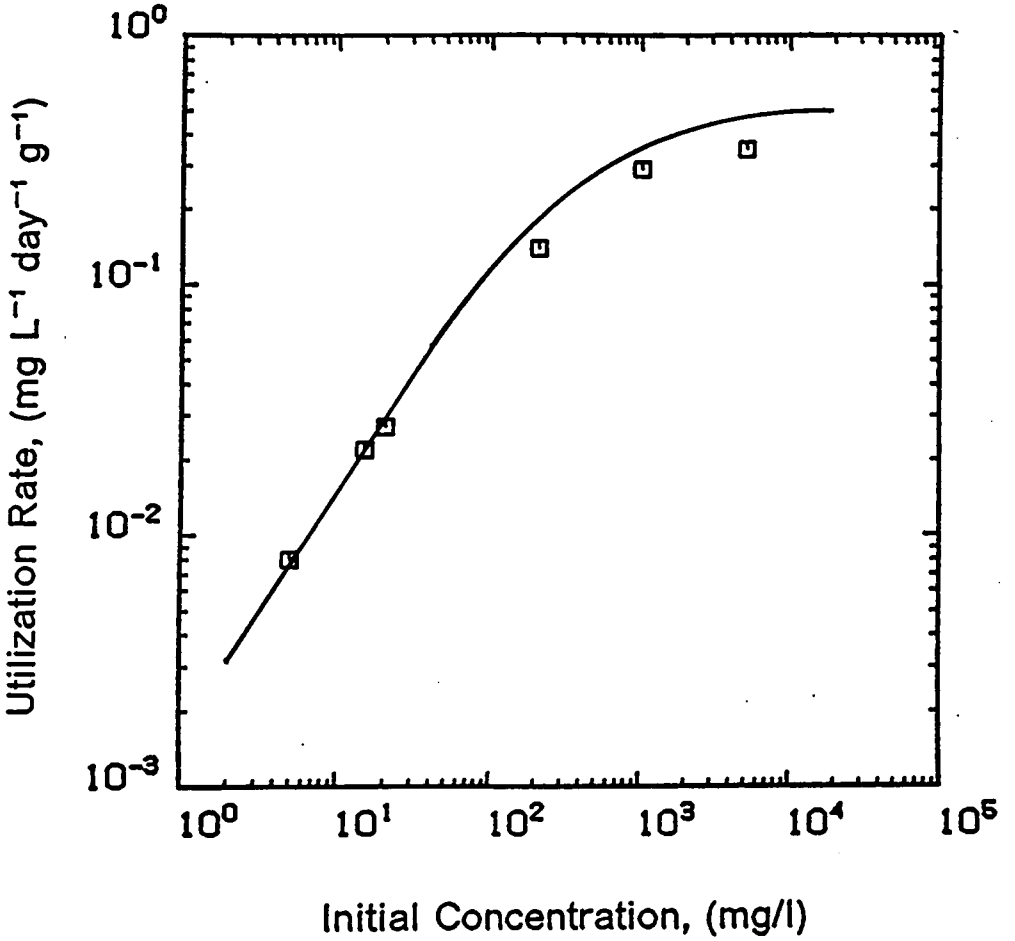

Figure 48. Determination of TBA utilization response in relation to initial concentration for Blacksburg soil (site 1, 15 feet) with molybdate.

g soil^{-1}. These values were used to contruct the line on Figure 48. By combining Figures 46, 47 and 48, TBA utilization with and without molybdate can be compared to the response of the Newport News soil (Figure 49). At high concentrations, the TBA degradation rate was similar for all three cases. As the initial concentration was decreased, however, the degradation rate for the molybdate dosed microcosms became increasingly greater than the unamended Blacksburg soil and the rate in the Newport News soil was greater than either of the Blacksburg soils. This relationship is reflected in the K_s for each system. These values have been summarized in Table 8. Systems in which the maximum substrate utilization rate is constant, but the half saturation constant is different is typical of a system which is controlled by a competitive inhibition. A double reciprocal plot of the three cases yields a similar y-intercept ($\frac{1}{k}$) but a different slope ($\frac{K_s}{k}$) (Figure 50). As the slope of the line on the double reciprocal plot (corresponding to a higher K_s) increases, the greater is the level of inhibition or concentration of the inhibitor. The question which needs to be answered, therefore, is what is in competition. There is no direct evidence in this study which would definitely answer this question. A possible candidate based on previous discussion would be hydrogen. This would presume that sulfate reducing organisms cannot effectively degrade TBA but that methanogens can use TBA given a supply of hydrogen. More research is necessary to answer this question.

In microcosms containing both methanol and TBA, a diauxic response was observed in the Newport News soil. As shown in Figure 51a, TBA degraded slowly until the methanol had been consumed. In the Blacksburg soil, however, TBA did not significantly degrade even after the methanol had been used (Figure 52a). About 25 mg/l methanol degraded in 15 days, but only about 4 mg/l of TBA had been degraded after 175 days. When molybdate was added to Blacksburg soil containing methanol and TBA, however, a diauxic growth pattern was observed (Figure 52b). Approximately 20 mg/l TBA was degraded to an undetectable level in about 175 days once the methanol was used. Molybdate did not affect the diauxic pattern in the Newport News soil (Figure 51b)

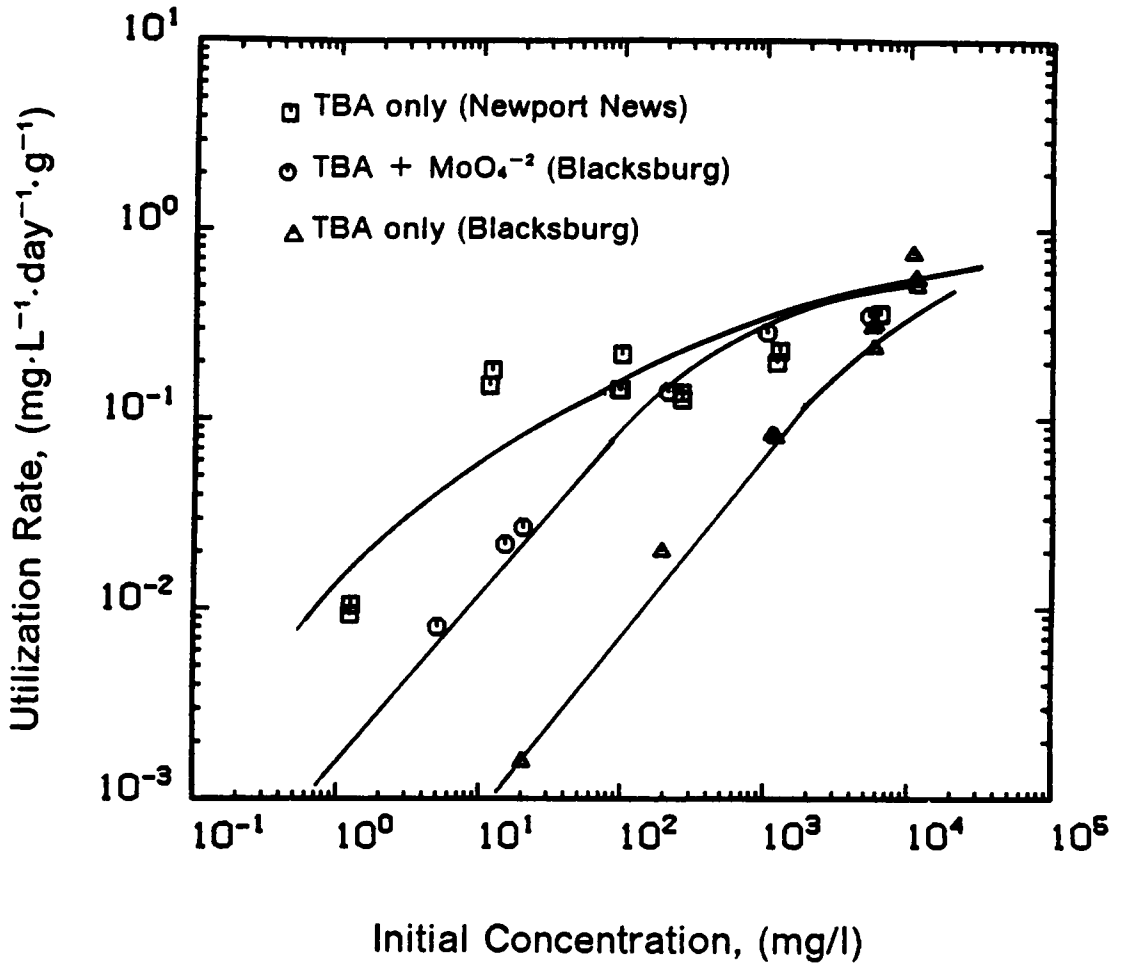

Figure 49. Composite of the TBA utilization response in the Newport News soil and the Blacksburg soil with and without molybdate.

Table 8. Summary of kinetic coefficients for TBA utilization in three soil systems.

System	K_s $\frac{mg}{L}$	k $\frac{mg}{L \cdot d \cdot g \cdot soil}$
Blacksburg (unamended)	9600	0.83
Blacksburg ($+ MoO_4^{-2}$)	365	0.55
Newport News	19	0.36

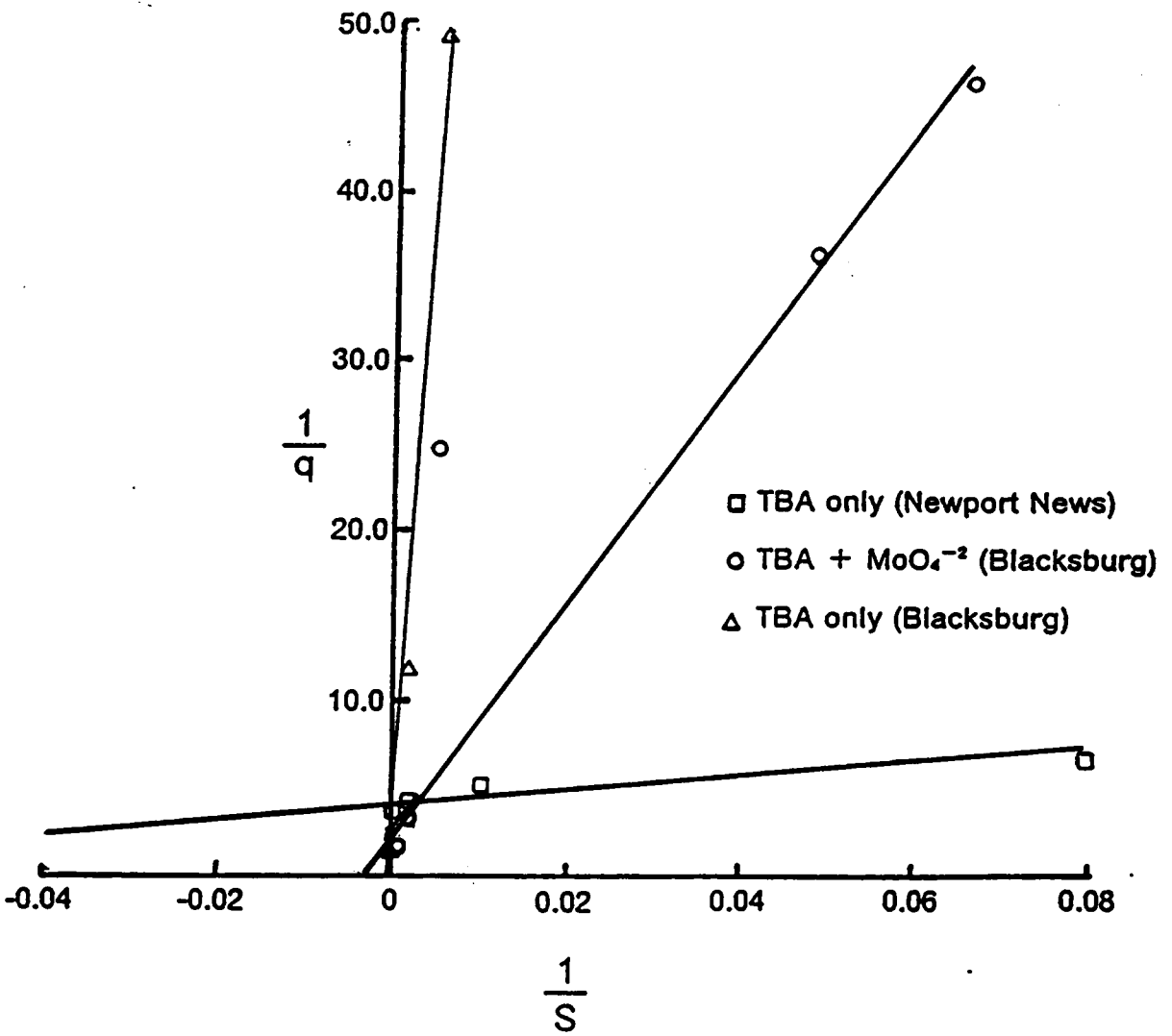

Figure 50. Lineweawer-Burke reciprocal modification of TBA utilization in Newport News soil and Blacksburg soil with and without molybdate.

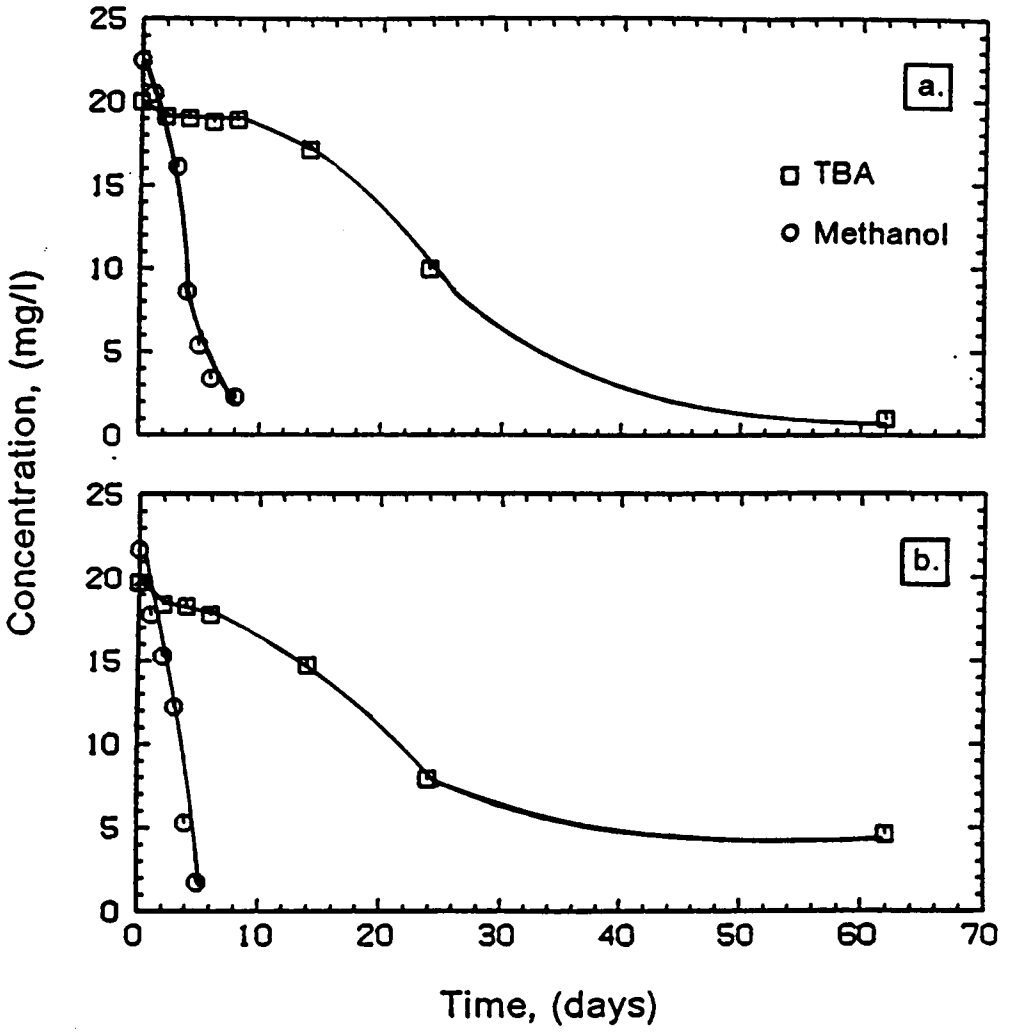

Figure 51. TBA biodegradation in the presence of methanol in Newport News soil (a: without molybdate; b: with molybdate).

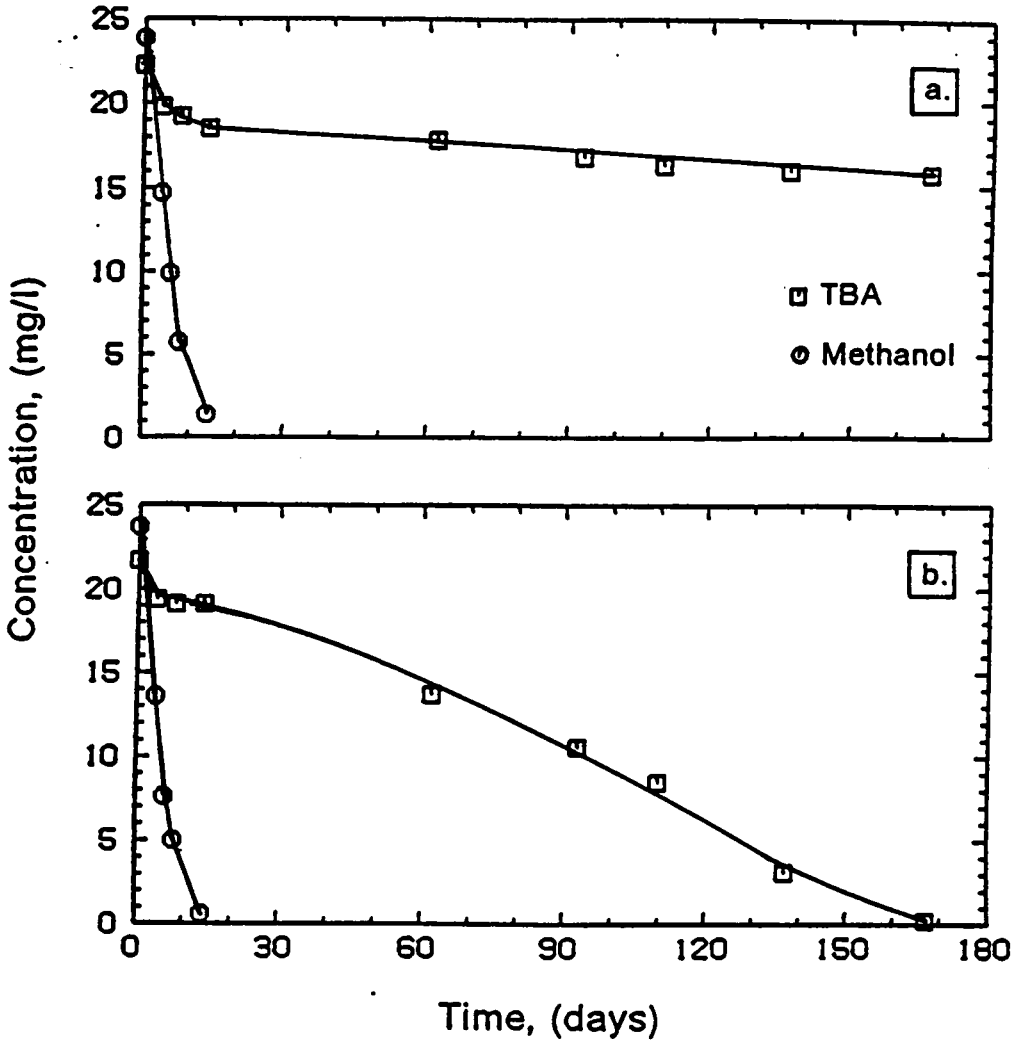

Figure 52. TBA biodegradation in the presence of methanol in Blacksburg soil (site 1, 15 feet) (a: without molybdate; b: with molybdate).

Diauxic substrate removal was first observed in the early 1900's, however, it was not until the 1940's that Monod (1949) reported the first quantitative data and used the term "diauxie" growth. In Monod's experiments, pure cultures of *B. subtilus* and *E. coli* exhibited double or triple growth cycles when grown in a mixed carbohydrate media. This was attributed to sequential substrate utilization whereby the first or primary substrate was degraded prior to the ulilization of the secondary substrate. It is generally accepted that the compound which can be used first is the primary substrate.

Diauxic growth has also been observed in heterogeneous bacterial populations. Gaudy (1962) reported that cells originating from activated sludge and grown on sorbitol, exhibited preferential substrate utilization when placed in a media containing glucose and sorbitol. Sorbitol was not used until the glucose had been exhausted. Similar results were obtained by Prakasam and Dondero (1964) using activated sludge.

Several mechanisms may account for a diauxic response. One widely accepted explanation is catabolite repression, whereby the presence of the primary substrate inhibits the production of enzymes needed to catalyze the degradation of the secondary substrate. Gaudy (1980) summarized the steps in catabolite repression as follows. The primary substrate is metabolized resulting in rapid growth and production of enzymes necessary for the degradation of the first substrate. The available cyclic AMP which is necessary for transcription of enzymes is decreased preventing synthesis of enzymes in the secondary substrate utilization pathway. Once the primary substrate has been consumed, the level of c-AMP increased, enzymes for the secondary substrate are produced and a second growth phase occurs. This explanation would suggest that the substrate which can be degraded the fastest and consequently compete for c-AMP will be the primary substrate.

A second possible mechanism for diauxic growth is catabolite inhibition in which the primary substrate inhibits the activity but not the production of enzymes in the secondary pathway. Gaudy, et al. (1963) reported that cells grown on a secondary substrate ceased using this substrate when a primary substrate was introduced. If catabolite repression was the only mechanism involved, pre-

synthesized enzymes should have continued to degrade the secondary substrate even after the primary substrate was added.

The diauxic substrate utilization pattern observed in the Newport News soil was not a surprising result considering this soils capacity to degrade TBA. Similar results were obtained by Goldsmith (1985) using soil from Williamsport, Pennsylvania. In Goldsmith's study, approximately 6 mg/l TBA degraded to below the detection limit in about 12 days, whereas, a similar TBA concentration required almost 50 days in the presence of 100 mg/l methanol. TBA degradation did not begin until the methanol had been consumed. Likewise, the absence of a diauxic response in unamended Blacksburg soil was not unexpected since TBA degraded slowly in microcosms which did not contain methanol. The diauxic response observed in the Blacksburg soil amended with molybdate indicated that enzyme systems are present in this soil for TBA degradation.

4.8. Thermodynamic Comparison

The free energies involving reactions with the four primary electron acceptors, oxygen, nitrate (to N_2), sulfate and carbon dioxide, and the straight chained alcohol series were calculated for comparisons with the measured substrate utilization rates for each soil. These calculations are contained in Appendix C. The free energies for the electron donor half reactions were determined assuming unit activities for all species except hydrogen which was given a value of 10^{-7} representing neutral water. By convention, the free energy of an electron at unit activity is zero. The free energy of formation for each compound was obtained from Lange's Handbook of Chemistry (1972). As Figure 53 shows, the free energy for the reaction did not correlate with the biodegradation rates in the Blacksburg and Newport News soils. The free energies per mole of substrate increased as the alcohol carbon number increased. The biodegradation rates, however, increased from methanol to ethanol, but decreased from ethanol to pentanol. The free energy values used to construct these figures are given in Tables 9 and 10. Free energy involving electron donor and electron acceptor half reactions cannot be used to estimate biodegradation rates.

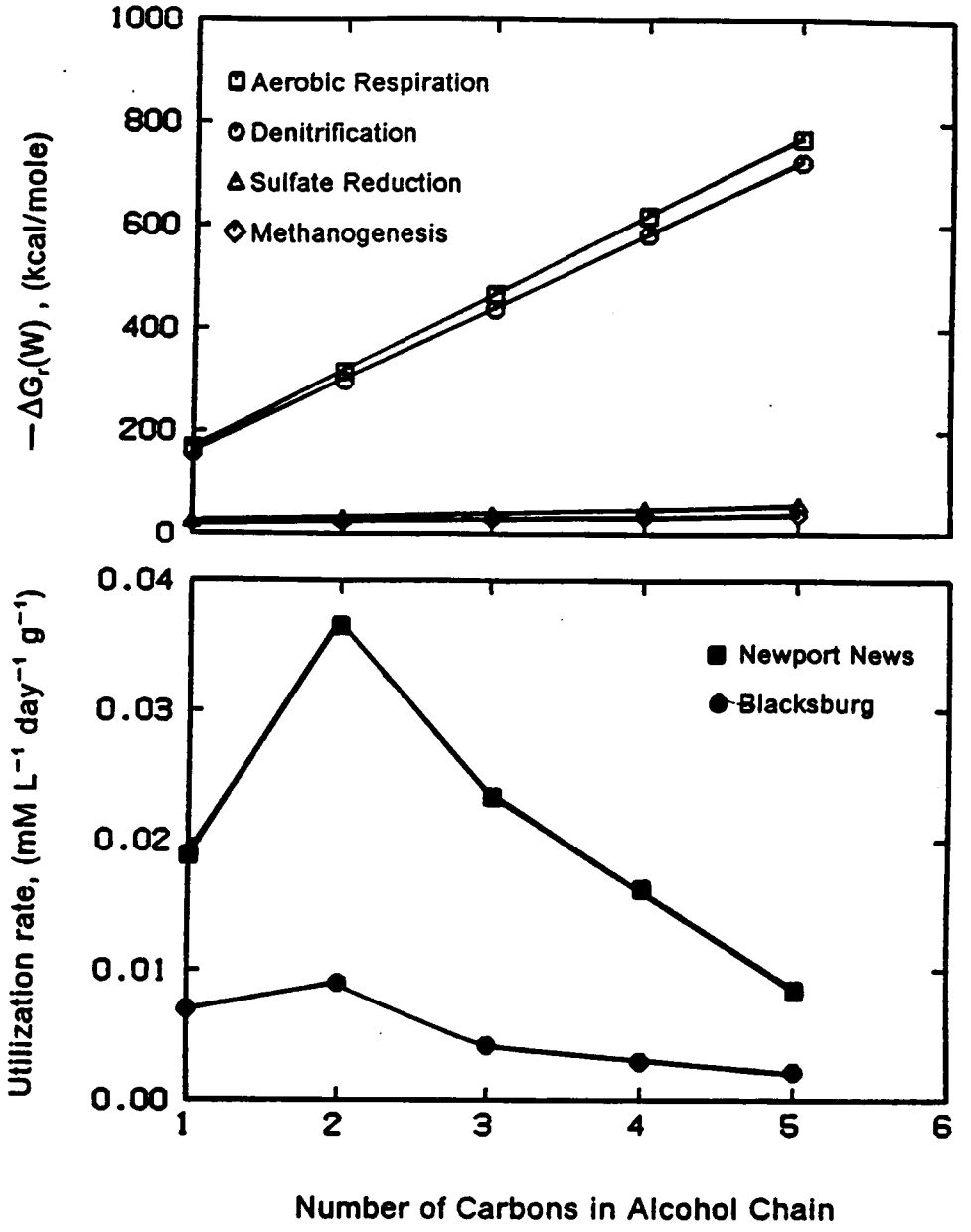

Figure 53. **Biodegradation rates and free energies for the reactions involving the straight chained alcohols in Blacksburg (site 1, 15 feet) and Newport News soils.**

Table 9. Free energy yield per mole substrate during aerobic respiration and nitrate reduction

Reaction	$G_r(W)$ kcal/mole
$CH_3OH + 1.5O_2 = CO_2 + 2H_2O$	-166.96
$CH_3CH_2OH + 3O_2 = 2CO_2 + 3H_2O$	-315.38
$CH_3(CH_2)_2OH + 4.5O_2 = 3CO_2 + 4H_2O$	-466.40
$CH_3(CH_2)_3OH + 6O_2 = 4CO_2 + 5H_2O$	-618.50
$CH_3(CH_2)_4OH + 7.5O_2 = 5CO_2 + 6H_2O$	-769.20
$C(CH_3)_3OH + 6O_2 = 4CO_2 + 5H_2O$	-613.20
$C_6H_5OH + 7O_2 = 6CO_2 + 3H_2O$	-720.94
$CH_3OH + 1.2NO_3^- + 1.2H^+ = CO_2 + 0.6N_2 + 2.6H_2O$	-157.67
$CH_3CH_2OH + 2.4NO_3^- + 2.4H^+ = 2CO_2 + 1.2N_2 + 4.2H_2O$	-296.82
$CH_3(CH_2)_2OH + 3.6NO_3^- + 3.6H^+ = 3CO_2 + 1.8N_2 + 5.8H_2O$	-438.55
$CH_3(CH_2)_3OH + 4.8NO_3^- + 4.8H^+ = 4CO_2 + 2.4N_2 + 7.4H_2O$	-581.38
$CH_3(CH_2)_4OH + 6NO_3^- + 6H^+ = 5CO_2 + 3N_2 + 9H_2O$	-722.79
$C(CH_3)_3OH + 4.8NO_3^- + 4.8H^+ = 4CO_2 + 2.4N_2 + 7.4H_2O$	-576.00
$C_6H_5OH + 5.6NO_3^- + 5.6H^+ = 6CO_2 + 2.8N_2 + 5.8H_2O$	-677.63

Table 10. Free energy yield per mole substrate for sulfate reduction and methanogenesis.

Reaction	$\Delta G_r(W)$ kcal/mole
$CH_3OH + 0.75SO_4^{-2} + 1.125H^+ = CO_2 + 0.375H_2S + 0.375HS^- + 2H_2O$	-24.40
$CH_3CH_2OH + 1.5SO_4^{-2} + 2.25H^+ = 2CO_2 + 0.75H_2S + 0.75HS^- + 3H_2O$	-30.26
$CH_3(CH_2)_2OH + 2.25SO_4^{-2} + 3.375H^+ = 3CO_2 + 1.125H_2S + 1.125HS^- + 4H_2O$	-38.72
$CH_3(CH_2)_3OH + 3SO_4^{-2} + 4.5H^+ = 4CO_2 + 1.5H_2S + 1.5HS^- + 5H_2O$	-48.26
$CH_3(CH_2)_4OH + 3.75SO_4^{-2} + 5.625H^+ = 5CO_2 + 1.875H_2O + 1.875HS^- + 6H_2O$	-56.40
$C(CH_3)_3OH + 3SO_4^{-2} + 4.5H^+ = 4CO_2 + 1.5H_2S + 1.5HS^- + 5H_2O$	-42.96
$C_6H_5OH + 3.5SO_4^{-2} + 5.25H^+ = 6CO_2 + 1.75H_2S + 1.75HS^- + 3H_2O$	-55.66
$CH_3OH = 0.25CO_2 + 0.75CH_4 + 0.5H_2O$	-20.33
$CH_3CH_2OH = 0.5CO_2 + 1.5CH_4$	-22.13
$CH_3(CH_2)_2OH + 0.5H_2O = 0.75CO_2 + 2.25CH_4$	-26.51
$CH_3(CH_2)_3OH + H_2O = CO_2 + 3CH_4$	-31.99
$CH_3(CH_2)_4OH + 1.5H_2O = 1.25CO_2 + 3.75CH_4$	-36.06
$C(CH_3)_3OH + H_2O = CO_2 + 3CH_4$	-26.29
$C_6H_5OH + 4H_2O = 2.5CO_2 + 3.5CH_4$	-36.40

As previously discussed, the work of McCarty (1969, 1972, and 1975) developed thermodynamic correlations between free energy, cell growth yield and substrate utilization rate. The following expressions were given:

$$\frac{dF}{dt} = \frac{dX_a/dt + bX_a}{a_e} \tag{4.8a}$$

where:

$\frac{dF}{dt}$ = substrate utilization rate, mass volume^{-1} time^{-1}

$\frac{dX_a}{dt}$ = biomass growth rate, mass volume^{-1} time^{-1}

X_a = biomass concentration, mass volume^{-1}

b = biomass decay coefficient, time^{-1}

a_e = growth yield coefficient

The growth yield coefficient, a_e, was estimated from thermodynamic considerations as follows:

$$a_e = \frac{1}{1 + A} \tag{4.8b}$$

where:

A = the electron equivalents of substrate derived energy per electron equivalent of cells synthesized

$$A = -\frac{\Delta G_p/k^m + 7.5 + \Delta G_n/k}{k\Delta G_r} \tag{4.8c}$$

where:

$\Delta G_p = \Delta G°(W)_{ethanol} - \Delta G°(W)_{pyruvate}$

k = efficiency of electron transfer from the electron donor to acceptor

m = + 1 if ΔG_p is positive and -1 if ΔG_p is negative.

ΔG_r = the free energy released per electron equivalent of substrate oxidized

ΔG_n = the free energy to produce ammonia from inorganic nitrogen.

Substituting equation 4.8c into equation 4.8a and 4.8b yields:

$$\frac{dF}{dt} = (1 + A)(\frac{dX_a}{dt} + bX_a) \tag{4.8d}$$

The value of A and the substrate utilization rate, therefore, are positively correlated. If A increases, $\frac{dF}{dt}$ increases. In other words, the more energy derived from the electron donor to synthesize cells, the greater will be the substrate utilization rates. This relationship was tested for the observations made in this study.

Using ethanol and pentanol as electron donors and nitrate as the electron acceptor and the nitrogen source, A was calculated as follows. The free energy for the half reaction involving pyruvate was obtained from McCarty (1972). The electron transfer efficiencies (k) were assumed to equal 0.6. The value of A for ethanol can be determined using the following relationship:

$$A = -\frac{\Delta G_p/k^m + 7.5 + \Delta G_n/k}{k\Delta G_r} \tag{4.8e}$$

$\Delta G_r = -24.74$ kcal/mole electrons

$\Delta G_p = \Delta G^\circ(W)_{ethanol} - \Delta G^\circ(W)_{pyruvate} = 0.94$ kcal/mole electrons

$\Delta G_n = 4.17$ kcal/mole electrons

$$A = -\frac{0.94/0.6 + 7.5 + 4.17/0.6}{(0.6)(-24.74)} = 1.079$$

The value for pentanol is given as follows:

$\Delta G_r = -24.09$ kcal/mole electrons

$\Delta G_p = 1.58$ kcal/mole electrons

$\Delta G_n = 4.17$ kcal/mole electrons

$$A = -\frac{1.58/0.6 + 7.5 + 4.17/0.6}{(0.6)(-24.09)} = 1.182$$

For these compounds, therefore, the value of A for pentanol is greater than the value for ethanol. Equation 4.8d, therefore, would predict greater utilization rate for pentanol than for ethanol. The degradation rates, however, were less for pentanol than for ethanol in both the Blacksburg and Newport News soils. A more complete listing of the A values for each electron acceptor is given

in Table 11. For simplicity, the nitrogen source was assumed to be ammonia and the electron transfer efficiencies were equal to 0.6. Among the homologous alcohol series, the value of A increased as the carbon chain length increased. The value of A for TBA was greater than that for pentanol. Phenol values were less than pentanol but greater than 1-butanol. The values of A which are based on the free energy of half reactions involving an electron donor and electron acceptor do not predict the degradation rates in the subsurface systems for Blacksburg and Newport News. This may be due to the fact that the subsurface bacteria were not cultured on the test compounds.

McCarty and Smith (1986) have described hydrogen-dependent energetics which exert control over anaerobic processes. As described previously, the methanogenesis of complex organics involves a symbiotic relationship between three bacteria species. The first species degrades complex organics into acetate and hydrogen (acetogenesis). Hydrogen and carbon dioxide are combined to form methane by another species called obligate proton reducers. The third species splits acetate into carbon dioxide and methane. The first reaction involving the production of acetate and hydrogen has a positive free energy when the reactants and products are at unit activity and the pH is 7. As the product concentration is reduced, however, the free energy of the reaction becomes negative and the reaction becomes more favorable. The activity of the second and third species of bacteria in reducing the H_2 and acetate concentration, therefore, is necessary to provide a suitable environment for the acetogens.

According to McCarty and Smith (1986), the H_2 concentration is more significant in anaerobic process control than is acetate. Hydrogen levels in anaerobic treatment of municipal wastewater typically range from 10^{-8} M to 10^{-5} M, whereas, acetate concentration is much greater, ranging from 10^{-4} M to 10^{-1} M. In addition, H_2 partial pressure can change rapidly because of its high turnover rate. The effect of H_2 concentration on the free energy of reaction involving the conversion of a complex substrate to methane is illustrated below with ethanol serving as the initial substrate (McCarty and Smith, 1986). All reactants and products were assumed to be at unit activity except the following: $[CH_4] = 0.7$ atm; $[CO_2] = 0.3$ atm; pH = 7.0. Free energy values for each compound were obtained from Lange's Handbook of Chemistry (1972).

Table 11. Values for A which relate the substrate derived energy with the amount of cells synthesized.

Substrate	A			
	O_2	NO_3^-	SO_4^{-2}	CO_2
Methanol	0.43	0.45	2.92	3.51
Ethanol	0.58	0.61	6.00	8.21
Propanol	0.62	0.66	7.51	10.98
1-Butanol	0.64	0.68	8.22	12.43
Pentanol	0.66	0.70	8.98	14.07
TBA	0.67	0.71	9,57	15.43
Phenol	0.65	0.69	8.31	12.68

1. Acetogenesis

$$CH_3CH_2OH_{(aq)} + H_2O_{(l)} = CH_3COO^-_{(aq)} + H^+_{(aq)} + 2H_{2(g)}$$

$$\Delta G° = -88.29 - (-41.63 + (-56.69)) = 10.03 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[H_{2(g)}]^2[H^+_{(aq)}][CH_3COO^-_{(aq)}]}{[CH_3CH_2OH_{(aq)}]}$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CH_3COO^-_{(aq)}] - \ln[CH_3CH_2OH_{(aq)}])$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] + \ln[H^+_{(aq)}])$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] - 16.12)$$

2. Proton Reduction

$$2H_{2(g)} + \frac{1}{2}CO_{2(g)} = \frac{1}{2}CH_{4(g)} + H_2O_{(l)}$$

$$\Delta G° = -56.69 + (\tfrac{1}{2})(-12.15) - (\tfrac{1}{2})(-94.26) = -15.64 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}]^{\frac{1}{2}}}{[H_{2(g)}]^2[CO_{2(g)}]^{\frac{1}{2}}}$$

$$\Delta G = -15.64 + 0.592(\tfrac{1}{2}\ln[CH_{4(g)}] - 2\ln[H_{2(g)}] - \tfrac{1}{2}\ln[CO_{2(g)}])$$

$$\Delta G = -15.64 + 0.592(\tfrac{1}{2}\ln(0.7) - 2\ln[H_{2(g)}] - \tfrac{1}{2}\ln(0.3))$$

$$\Delta G = -15.64 + 0.592(0.424 - 2\ln[H_{2(g)}])$$

3. Methane and Carbon Dioxide Production from Acetate

$$CH_3COO^-_{(aq)} + H^+_{(aq)} = CH_{4(g)} + CO_{2(g)}$$

$$\Delta G° = -12.15 + (-94.26) - (-88.29) = -18.12 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}][CO_{2(g)}]}{[CH_3COO^-_{(aq)}][H^+_{(aq)}]}$$

$$\Delta G = -18.19 + 0.592(\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3COO^-_{(aq)}] - \ln[H^+_{(aq)}])$$

$$\Delta G = -18.19 + 0.592(\ln(0.7) + \ln(0.3) - \ln(10^{-7}))$$

$$\Delta G = -9.50 \text{ kcal}$$

4. Net Reaction

$$CH_3CH_2OH_{(aq)} = \frac{3}{2}CH_{4(g)} + \frac{1}{2}CO_2(g)$$

$$\Delta G^\circ = \frac{1}{2}(-94.26) + \frac{3}{2}(-12.15) - (-41.63) = -23.73 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^{\frac{3}{2}}[CO_{2(g)}]^{\frac{1}{2}}}{[CH_3CH_2OH_{(aq)}]}$$

$$\Delta G = -23.73 + 0.592(\frac{3}{2}\ln[CH_{4(g)}] + \frac{1}{2}\ln[CO_{2(g)}] - \ln[CH_3CH_2OH])$$

$$\Delta G = -23.73 + 0.592(\frac{3}{2}\ln(0.7) + \frac{1}{2}\ln(0.3))$$

$$\Delta G = -24.40 \text{ kcal}$$

According to these calculations, the free energies associated with the production of carbon dioxide and methane from acetate and the net reaction are independent of the H_2 partial pressure. On the other hand, the free energy of the acetogenesis and proton reduction reactions are directly related to the partial pressure of hydrogen. Figure 54 shows the relationship between the free energy of the acetogenesis reaction involving ethanol and the partial pressure of hydrogen. The upper boundary of the partial pressure is defined by the point at which the free energy of the acetogenesis reaction becomes positive. The location of this boundary would, therefore, be a function of the substrate. For ethanol, this point is at a H_2 partial pressure of about 7×10^{-1} atm. under the assumed conditions. The lower boundary is located at the point where the free energy of the proton reduction reaction becomes positive. Since the initial substrate is not involved in this reaction, the location of this boundary is independent of the organic compound. At an H_2 partial pressure of about 2.3×10^{-6} atm., the free energy of the proton reduction reaction is positive. For ethanol, therefore, the acetogenesis reaction is favored when the H_2 partial pressure ranges from about 10^{-1} atm to 10^{-6} atm under the assumed conditions.

This procedure was repeated for propanol, 1-butanol and pentanol to determine the affect of H_2 concentration on the free energies of the compounds. To make these calculations, a knowledge of the degradation pathway was necessary. According to Eichler and Schink (1985), ethanol,

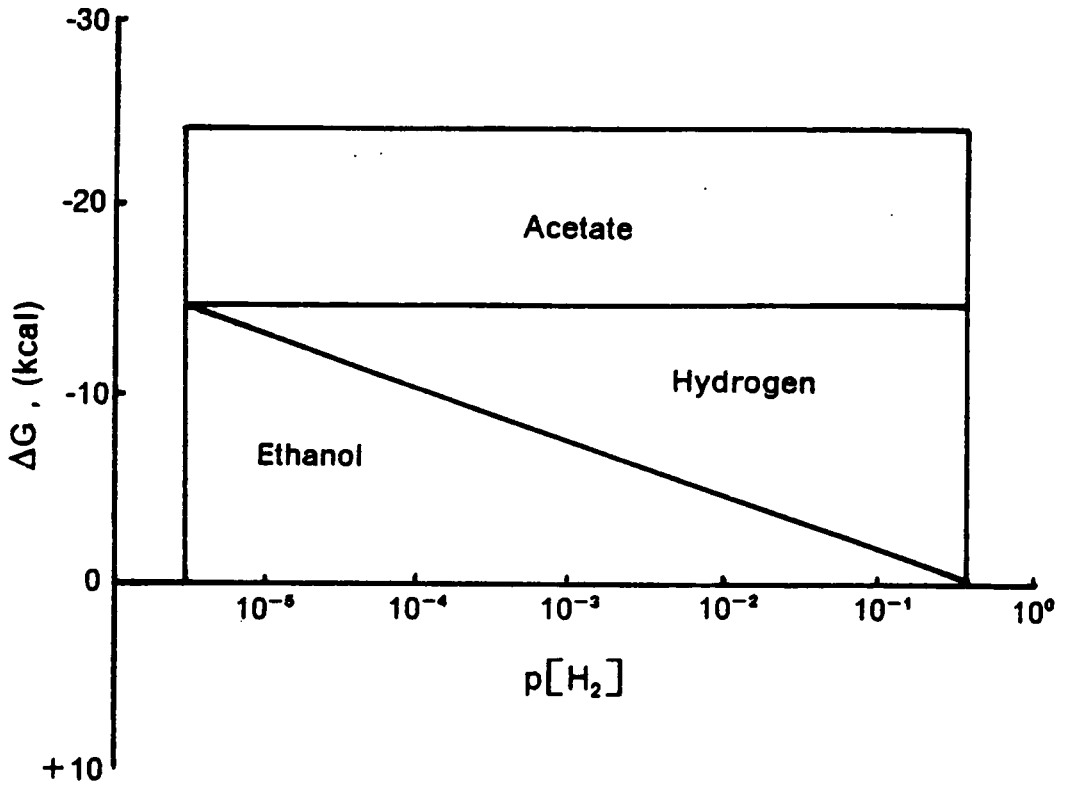

Figure 54. Free energy versus H_2 partial pressure for ethanol.

propanol, 1-butanol and pentanol were initially converted to acetate, propionate, butyrate and valerate, respectively, by a methanogenic consortium. Using this information and assuming that the fatty acids longer than two carbons were degraded by β-oxidation, the free energies of the acetogenic and methanogenic reactions were calculated. The calculations are contained in Appendix C. Methanol was not included in these calculations because it can be converted directly to methane. TBA was also not included with the other alcohols because no specific pathway could be found in the literature. From these calculations, Figure 55 was constructed in which the free energy of the acetogenic reaction of the initial substrate is related to acetate concentration and the H_2 partial pressure. As shown in Figure 55, the upper boundary of the H_2 partial pressure decreases as the alcohol carbon chain increases. Therefore, the range of H_2 partial pressures which result in a favorable free energy for the acetogenic reaction decreases as the alcohol carbon chain increases. The approximate H_2 ranges are 2×10^{-6} atm to 7×10^{-1} atm for ethanol; 2×10^{-6} atm to 2×10^{-3} atm for propanol; 2×10^{-6} atm to 7×10^{-4} atm for 1-butanol; and 2×10^{-6} to 3×10^{-4} atm for pentanol. Hydrogen concentrations, therefore, would be expected to exert greater control over hydrogen-dependent reactions as this range decreases. The degradation of pentanol, for example, would be more sensitive to the H_2 partial pressure than would ethanol.

4.9. Site Variations

From the results presented thus far in this study, factors which influence biodegradation can be described. First, the size of the bacterial population of a particular subsurface is not important in determining the biodegradability of a particular compound or the biodegradation rates. The bacterial numbers measured in the Blacksburg and Newport News soils were essentially equal. TBA, however, readily degaded in the Newport News soil, but persisted in the Blacksburg soil. In addition, the biodegradation rates of all compounds used in this study were greater in the Newport News soil than the Blacksburg soil. Obviously, environmental factors which influence the subsurface bacterial population and not the population size are important in predicting the biodegradation characteristics of a site. Table 13 compares some of these factors for each soil.

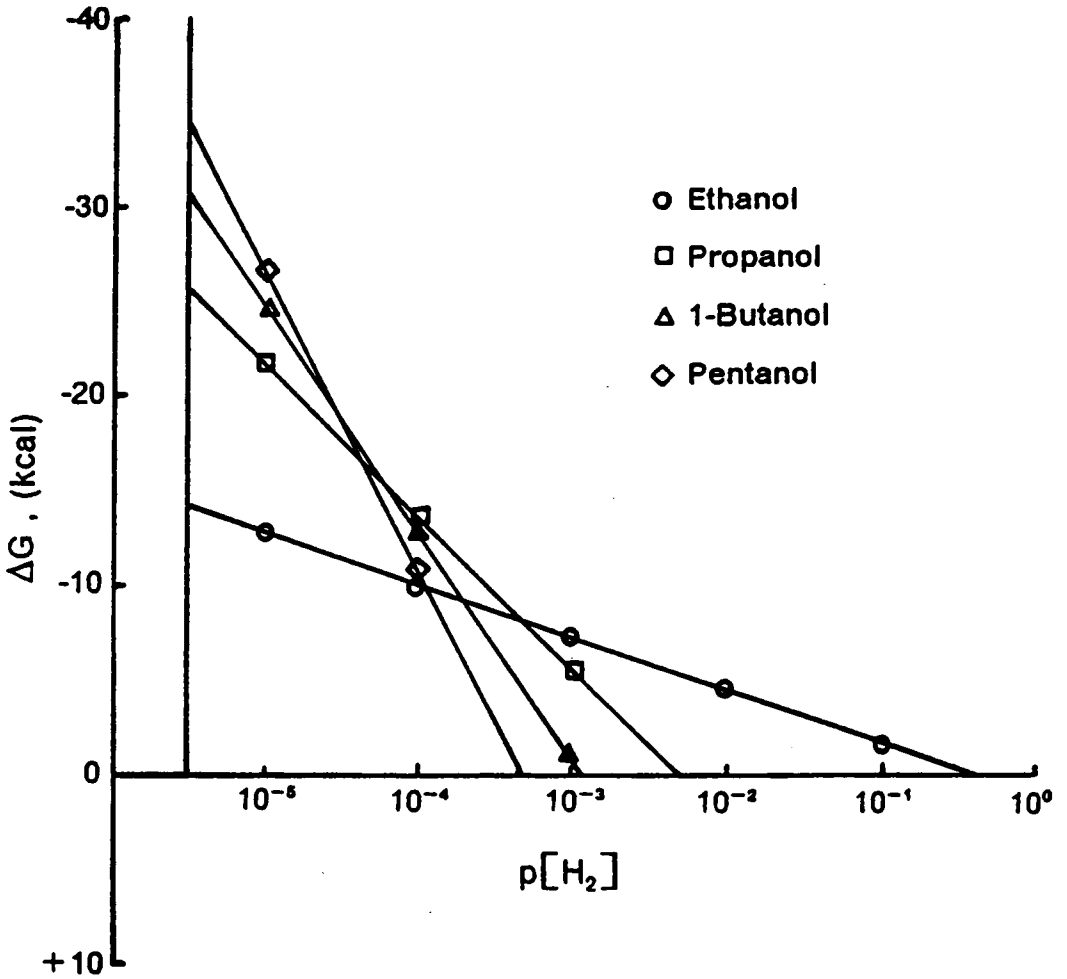

Figure 55. Free energy versus H_2 partial pressure for ethanol, propanol, 1-butanol and pentanol.

Table 12. Site differences related to degradation for Blacksburg and Newport News soils.

Condition	Blacksburg	Newport News
Degradation rates predicted from bacterial population	no	no
TBA degradation	slow	fast
Nitrate addition	no effect	increase rate
Molybdate addition to inhibit sulfate reduction	increase rate	no effect
BESA addition to inhibit methanogenesis	decrease rate	no effect

The free energy associated with the reaction of the homologous alcohol series and TBA with the four electron acceptor conditions was calculated to determine if free energy could predict the response of subsurface bacteria to organic chemicals. Free energy did not predict the rate of substrate utilization in the Blacksburg and Newport News soils. The biodegradation rates for the C2 through C5 alcohols decreased with carbon chain length, whereas, the free energy associated with the four electron acceptor conditions increased. In addition, the free energy associated with the biological degradation of 1-butanol and the tertiary form of butanol are very similar. For example, as shown in Table x, the free energy released in the methanogenic conversion of 1-butanol is -31.99 kcal/mole, while the free energy released in the reaction of TBA under the same conditions is -26.29 kcal/mole. The degradability of the two compounds, however, were strikingly different. 1-Butanol degraded at a rate of 3×10^{-3} mM day^{-1} g $soil^{-1}$ in Blacksburg soil while the TBA degradation rate was 1.9×10^{-5} mM day^{-1} g $soil^{-1}$. In the Newport News soil, degradation rate of 1-butanol was 1.7×10^{-2} mM day^{-1} g $soil^{-1}$ while the rate for TBA was 0.3×10^{-2} mM day^{-1} g $soil^{-1}$. This may be because the subsurface bacteria were not cultured on the test compounds and that the systems were not at steady state. These conditions, however, would be typical to subsurface environments which have been contaminated with anthropogenic chemicals. Aquifers containing a contaminated plume which moves with the flow of groundwater would continually expose unacclimated organisms. The results from studies which use cultured bacteria, then explain the results in terms of thermodynamics would not be applicable to the subsurface.

Chemical structure appears to determine the degradation rates in subsurface soils. As shown by the data for the homologous alcohol series and TBA, in general, the more complicated the chemical, the slower the degradation rate.

Based on the kinetic evaluation of TBA in the two soils, the degradation is controlled by ecological factors which predominate in the soil. TBA readily degraded in the Newport News soil. In this soil, various electron acceptor conditions operated simultaneously. In the Blacksburg soil, however, TBA degraded significantly only when sulfate reduction was inhibited by the presence of molybdate. A double reciprocal analysis of the rate data indicated that a competitive inhibition

resulted in slow TBA utilization. Evidence in the literature on the interactions between methanogenic bacteria and sulfate reducing bacteria mey indicate that the competition may be for hydrogen. It was speculated that this competition is more acute in degradation pathways such as DCP which require hydrogen in a structural position as opposed to the situation where hydrogen is involved in proton reduction. In the Newport News soil, the supply of hydrogen was sufficient to alleviate the competition. This may account for the visible signs of sulfate reduction and the chromatographic peak corresponding to methane in the same microcosms.

Chapter 5

Conclusions

The primary objective of this study was to explain the kinetics of TBA subsurface degradation which had been observed by Goldsmith (1985) and White (1986) in microcosms containing soil from Wayland, New York and Dumfries, Virginia. In each of these soils, TBA degradation was first order with respect to initial concentration. The significance of this finding was that low concentrations of TBA would be expected to persist almost indefinitely under natural conditions. This study was undertaken, therefore, in an attempt to explain this kinetic response and describe subsurface conditions which are important in establishing the kinetics. Other compounds were added as test substrates to further describe subsurface degradation and the conditions which influence the responses.

From the results obtained in this study, the following conclusions can be made:

1. TBA degradation in Blacksburg soil was slow following the same pattern as was observed by others for Dumfries, Virginia and Wayland, New York soil. In all cases, TBA degradation was first order with respect to initial concentration.

2. TBA degradation in the Newport News soil could be described by Monod kinetics.

3. The addition of molybdate to inhibit sulfate reduction increased the degradation rates for all the test compounds in the Blacksburg soil. The affect was more pronounced for compounds which required hydrogen in a structural position during degradation as opposed to those compounds which use hydrogen during proton reduction. In microcosms containing TBA and molybdate the response could be described by Monod kinetics.

4. The addition of BESA to inhibit methanogenesis in Blacksburg soil decreased the degradation rates for all the test compounds except TBA which was not measured. These effects were especially pronounced for the phenols.

5. The addition of the two metabolic inhibitors to the Newport News soil did not affect the degradation rates for except for BESA in microcosms containing the phenols. The degradation rates were slightly decreased under these conditions.

6. Nitrate addition to Blacksburg microcosms did not affect biodegradation rates for any of the test compounds presumably because there was not an active population of bacteria present which could use nitrate as an electron acceptor. Nitrate addition to the Newport News soil, however, increased the degradation rates.

7. TBA degradation rates in the Blacksburg soil in controlled by a competitive inhibition which is reduced in the presence of molybdate.

Chapter 6

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Appendix A
Blacksburg Biodegradation Data

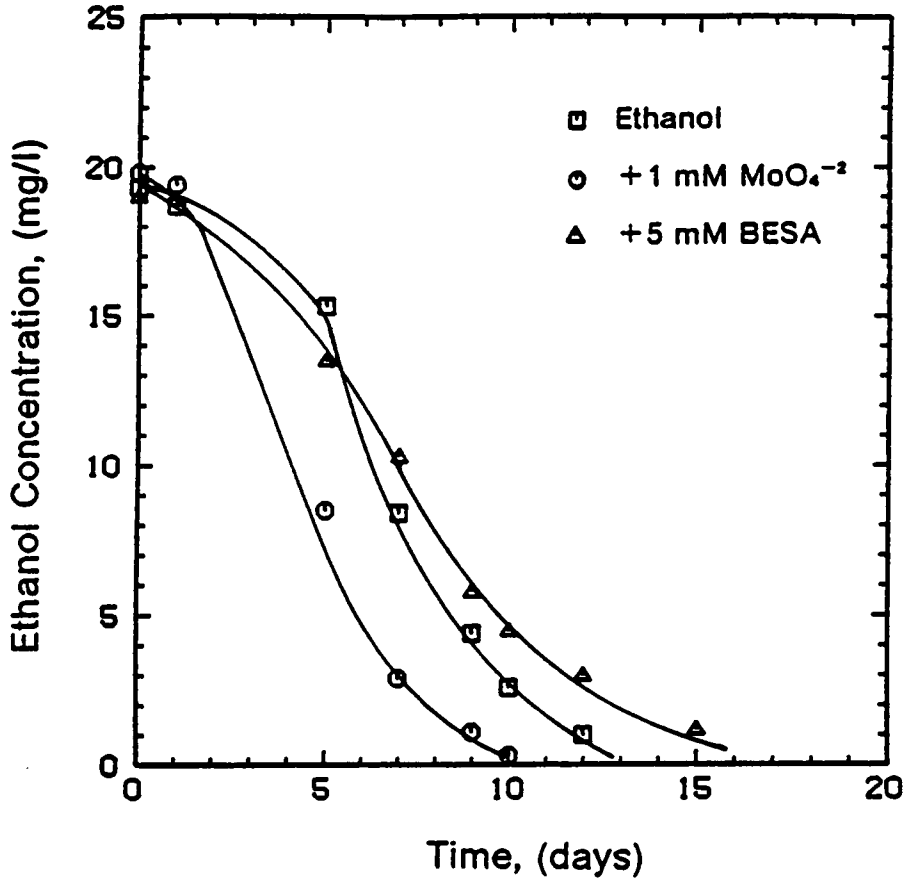

Figure 56. Ethanol biodegradation with molybdate and BESA in Blacksburg soil (site 1, 15 feet).

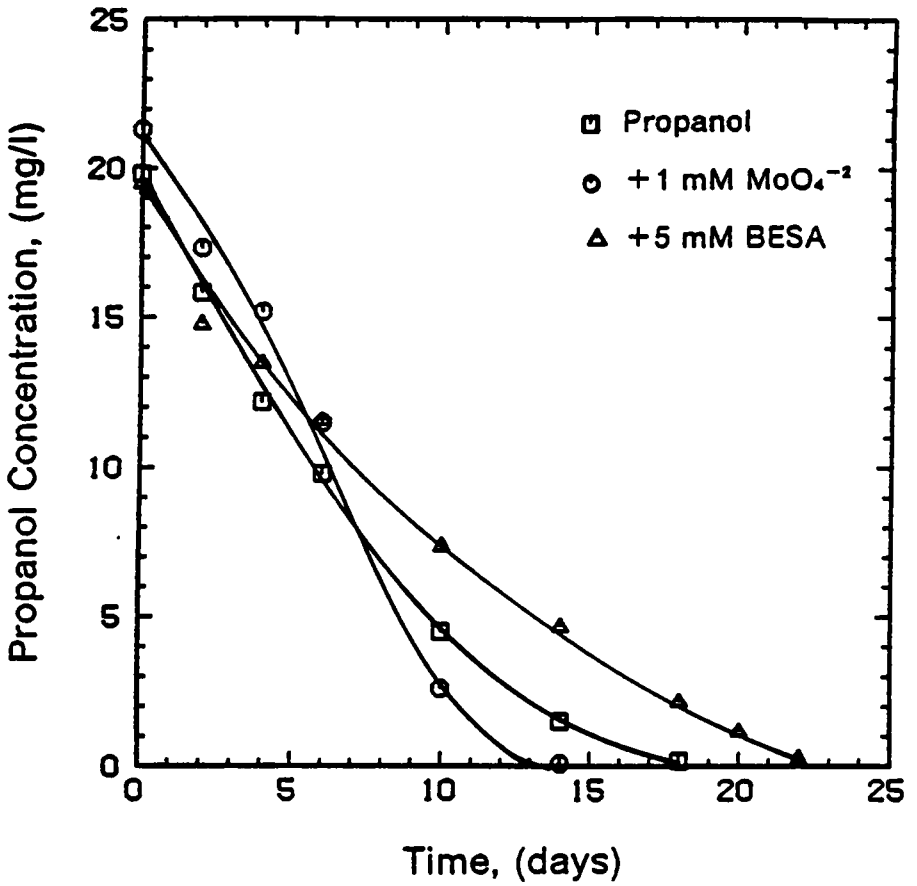

Figure 57. Propanol biodegradation with molybdate and BESA in Blacksburg soil (site 1, 15 feet).

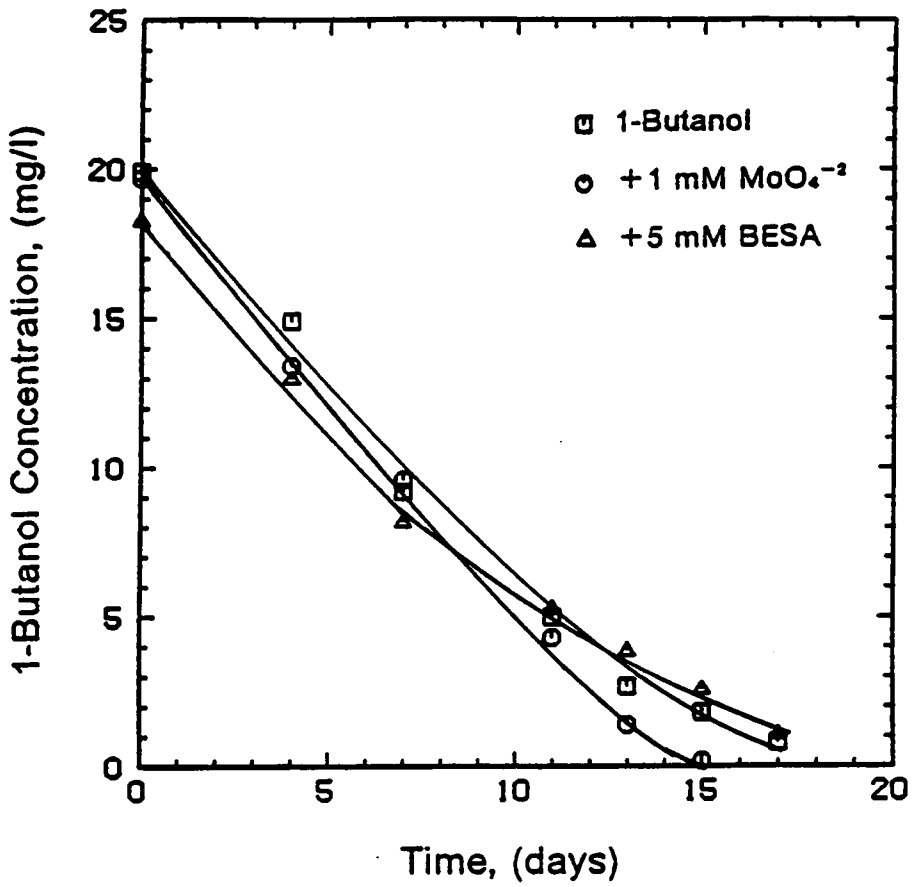

Figure 58. 1-Butanol biodegradation with molybdate and BESA in Blacksburg soil (site 1, 15 feet).

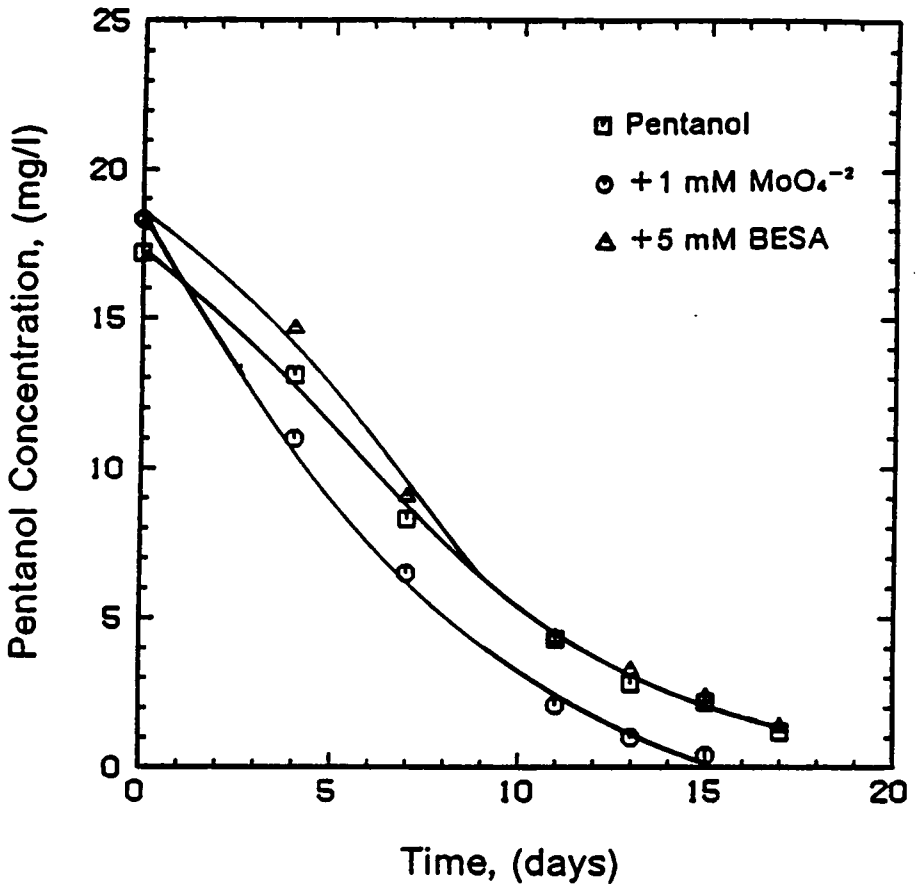

Figure 59. Pentanol biodegradation with molybdate and BESA in Blacksburg soil (site 1, 15 feet).

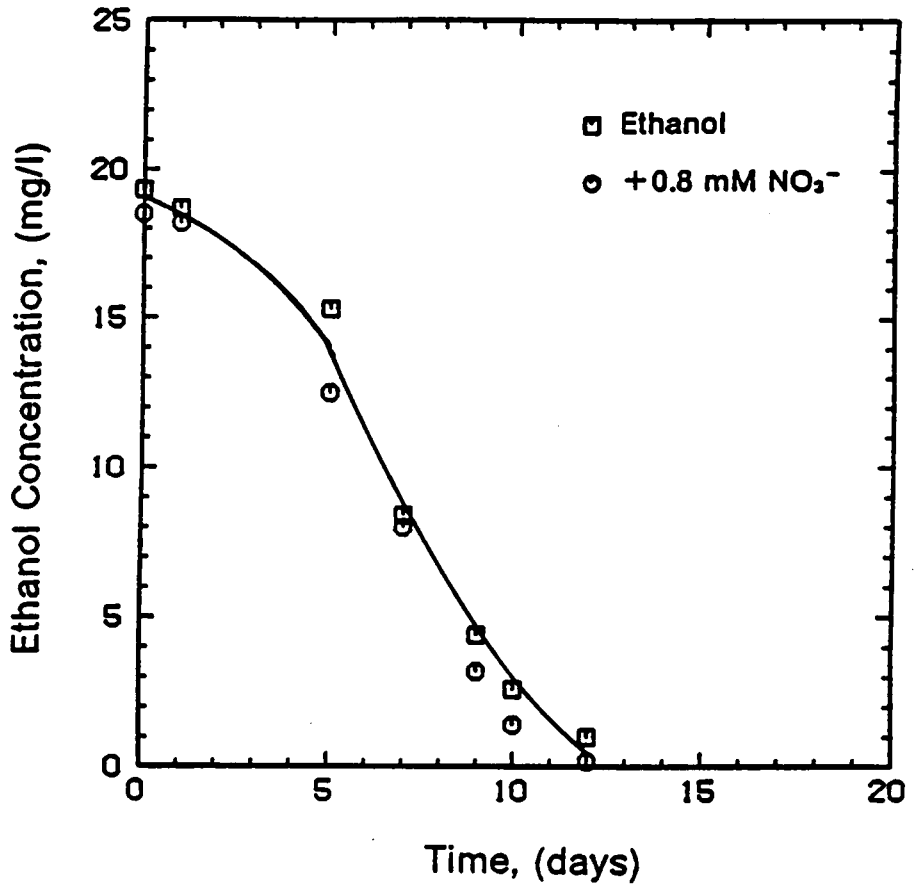

Figure 60. Ethanol biodegradation with nitrate in Blacksburg soil (site 1, 15 feet).

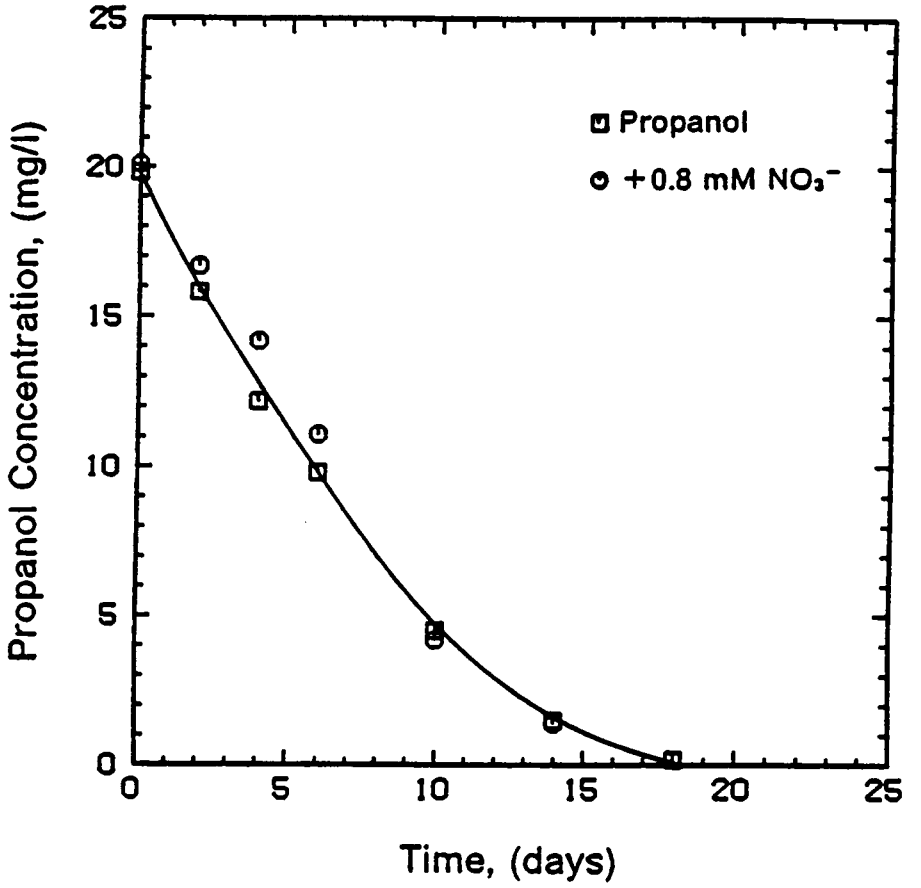

Figure 61. Propanol biodegradation with nitrate in Blacksburg soil (site 1, 15 feet).

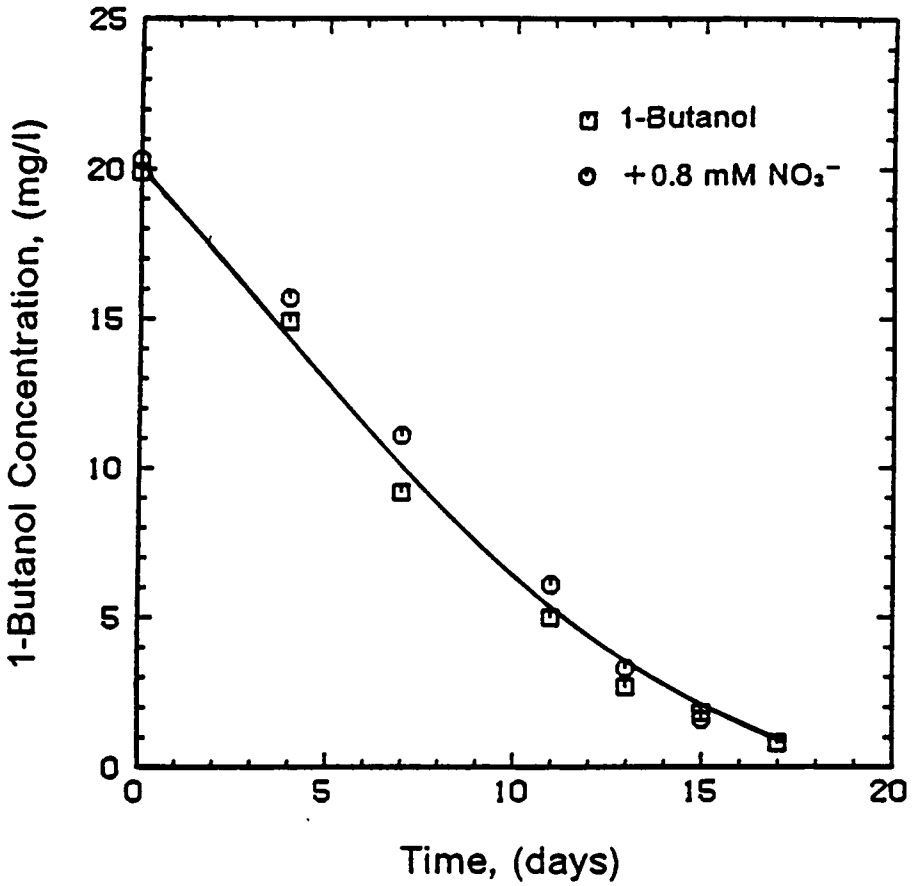

Figure 62. 1-Butanol biodegradation with nitrate in Blacksburg soil (site 1, 15 feet).

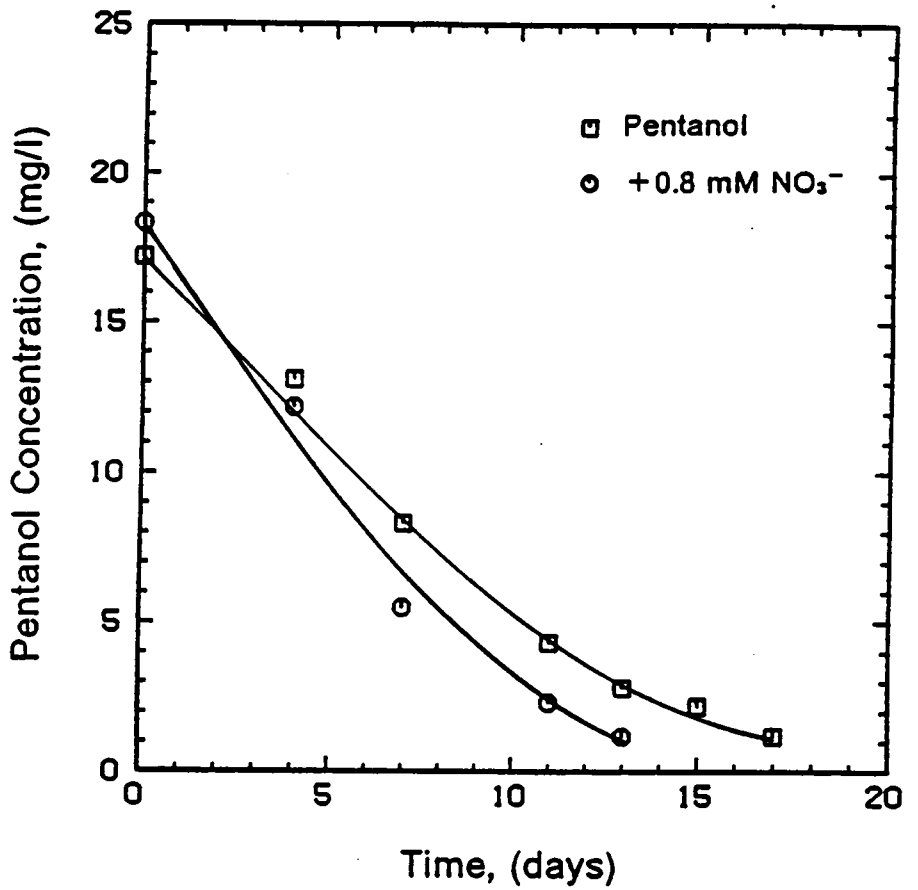

Figure 63. Pentanol biodegradation with nitrate in Blacksburg soil (site 1, 15 feet).

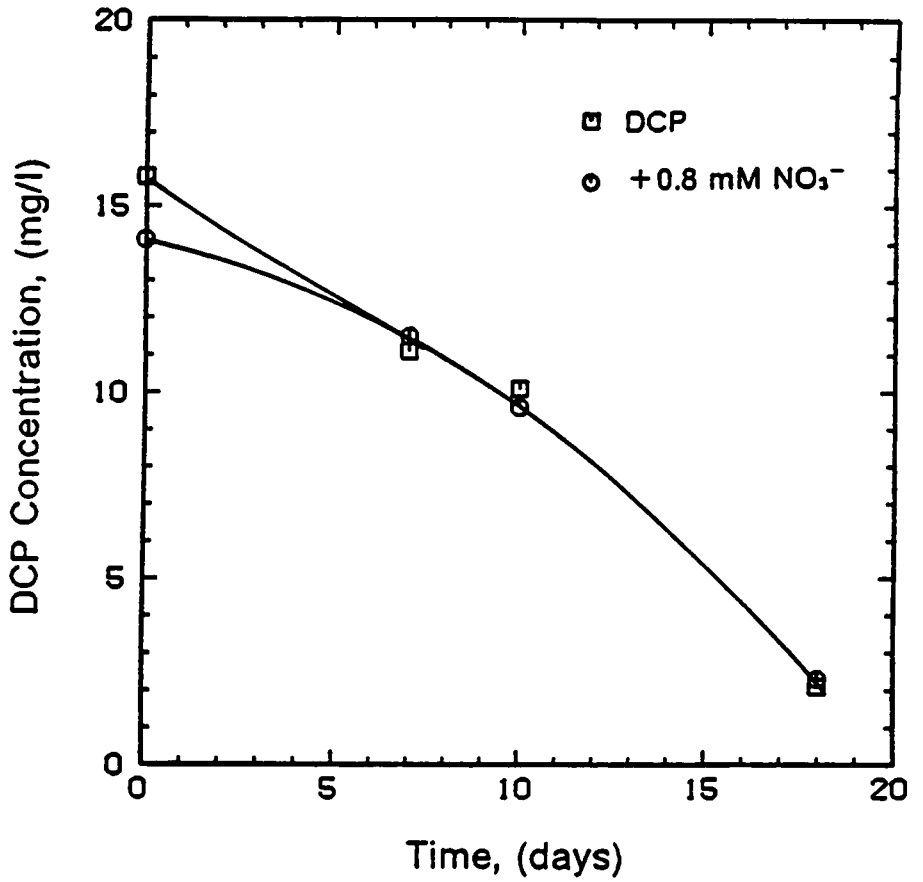

Figure 64. DCP biodegradation with nitrate in Blacksburg soil (site 1, 15 feet).

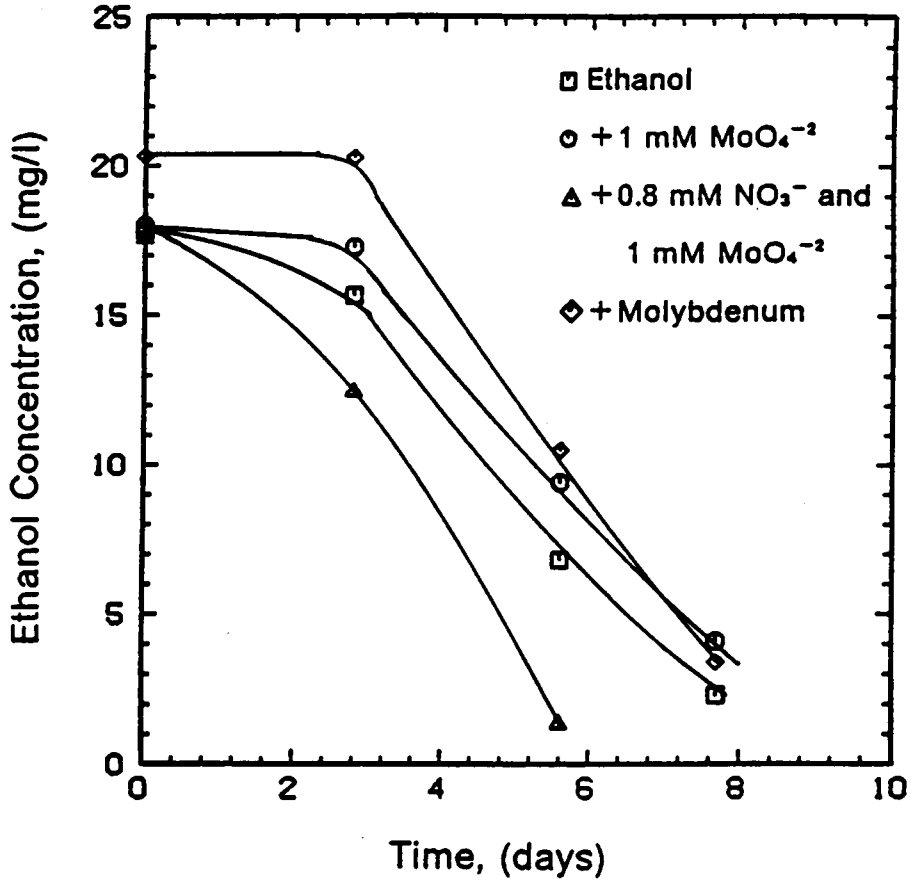

Figure 65. Ethanol biodegradation with molybdate, molybdate plus nitrate, and molybdenum in Blacksburg soil (site 2, 4 feet).

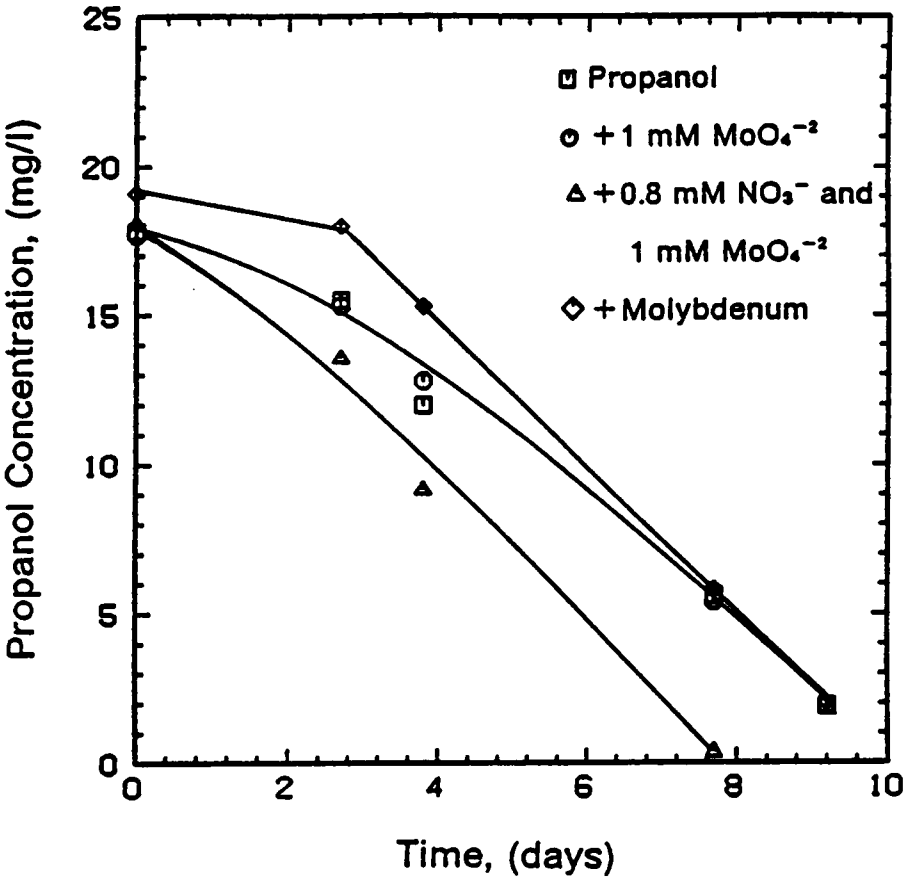

Figure 66. **Propanol biodegradation with molybdate, molybdate plus nitrate, and molybdenum in Blacksburg soil (site 2, 4 feet).**

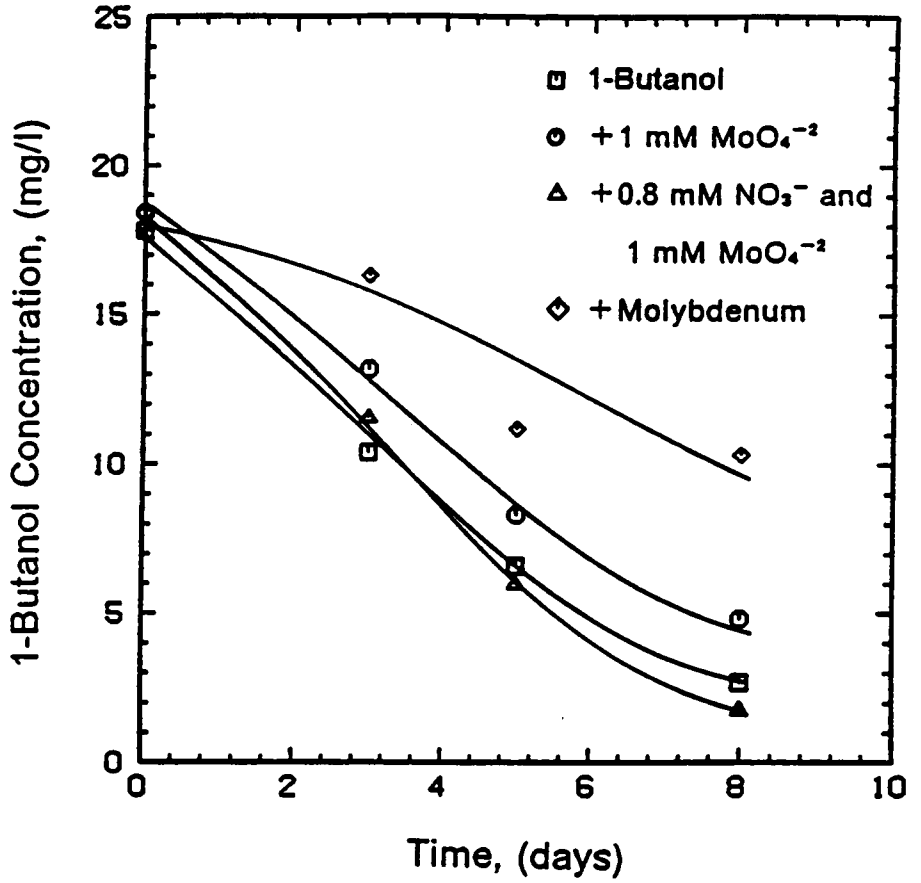

Figure 67. 1-Butanol biodegradation with molybdate, molybdate plus nitrate, and molybdenum in Blacksburg soil (site 2, 4 feet).

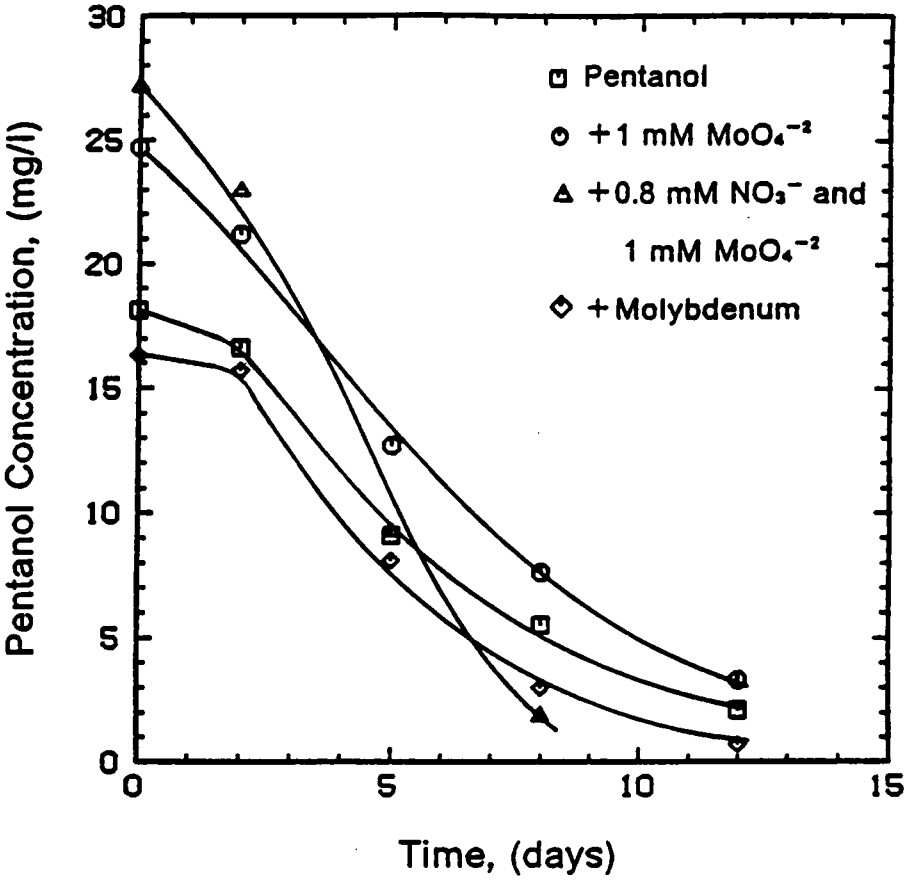

Figure 68. Pentanol biodegradation with molybdate, molybdate plus nitrate, and molybdenum in Blacksburg soil (site 2, 4 feet).

Table 13. TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	5600.1	5248.3	5337.5	5608.9
25	5322.0	4880.6	5224.3	5674.3
47	5167.4	4698.1	5201.0	5611.9
61	5093.7	4664.1	5139.9	5584.1
108	5041.8	4620.9	5072.4	--
231	4748.3	4269.0	4660.3	5502.0
348	4621.3	4166.7	4568.8	5500.0

Table 14. TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	1155.7	1025.1	1076.8	1255.4
25	1115.5	961.0	1072.9	1253.1
47	1065.4	916.4	1054.1	1231.8
61	1053.9	919.0	1037.9	1239.8
108	1024.3	842.8	962.5	--
348	922.3	787.1	931.6	1193.0

Table 15. TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	192.2	210.4	199.1	206.9
25	186.0	179.0	186.8	197.4
47	190.5	172.8	183.7	199.9
61	190.1	174.6	187.6	201.6
108	186.1	173.6	185.6	--
231	171.1	153.6	175.8	195.2
348	154.4	129.3	157.6	188.3

Table 16. TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	20.4	15.2	19.3	20.9
25	19.2	8.4	18.5	19.9
47	19.4	5.5	19.2	20.4
56	19.4	4.3	19.1	20.6
61	19.1	3.6	18.9	20.3
75	18.9	1.7	19.1	--
83	18.8	0.7	18.5	19.6
86	--	0.3	18.2	--
108	17.8	--	17.8	--
199	17.7	--	17.0	--
231	16.7	--	16.7	18.9
348	16.4	--	16.2	18.8

Table 17. TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	6.80	5.10	6.60	4.30
25	7.85	4.35	6.80	4.30
47	7.89	2.69	6.85	4.28
56	7.96	2.23	6.96	4.30
61	7.92	2.01	6.90	4.43
75	--	1.32	--	--
86	7.95	0.80	6.85	4.00
90	7.94	0.62	6.79	--
111	7.78	0.12	6.77	--
201	7.67	--	6.56	--
233	7.20	--	6.30	4.21

Table 18. TBA biodegradation in Blacksburg soil (incubated in anaerobic glove box).

Time (days)	TBA mg/l	Autoclaved Control	TBA mg/l	Autoclaved Control
0	8846.0	9066.4	4547.8	5124.1
14	8335.9	9012.4	4200.9	5186.8
28	7883.4	9027.4	4089.3	5145.9
41	7694.6	----	3849.3	5187.5
47	7567.9	----	3823.7	5123.3
77	7170.6	8819.4	3758.4	5142.2

Time (days)	TBA mg/l	Autoclaved Control	TBA mg/l	Autoclaved Control
0	864.5	843.0	174.2	166.8
14	808.6	---	162.9	182.5
28	788.7	866.3	158.4	184.9
41	---	---	157.6	182.6
47	768.7	822.0	156.3	181.9
77	737.9	809.4	153.0	182.0

Table 19. Methanol and TBA biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Alcohol Concentration, mg/l					
	Methanol	Methanol + MoO_4^{-2}	TBA	TBA + MoO_4^{-2}	Methanol/TBA	Methanol/TBA + MoO_4^{-2}
0	22.8	19.8	21.5	20.4	23.9/22.3	23.7/21.7
3	19.1	18.0	--	--	-- / --	-- / --
4	15.6	15.5	--	--	14.7/19.8	13.6/19.4
5	13.9	13.0	19.0	18.2	-- / --	-- / --
6	12.7	11.6	--	--	9.9/19.1	7.6/19.1
8	10.1	7.0	19.1	18.3	5.7/19.2	5.0/19.1
14	3.0	2.6	17.8	16.9	1.4/18.5	0.6/19.1
62	--	--	17.7	4.9	-- /17.8	-- /13.7
93	--	--	17.0	1.0	-- /16.8	-- /10.6
110	--	--	16.7	--	-- /16.3	-- / 8.5
137	--	--	16.6	--	-- /16.0	-- / 3.1
167	--	--	15.5	--	-- /15.8	-- / 0.2
240	--	--	16.5	--	-- /15.5	-- / --

Table 20. Methanol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Methanol Concentration, mg/l				
	Methanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	18.0	17.4	18.3	18.9	20.2
1	17.5	17.3	18.2	18.9	20.1
5	17.0	16.0	17.5	17.8	20.2
9	16.8	9.6	17.2	15.6	20.2
17	3.0	0.7	9.3	5.7	20.2
20	1.0	--	5.2	2.2	20.2
23	--	--	2.1	--	20.0

Table 21. Ethanol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Ethanol Concentration, mg/l				
	Ethanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	19.3	19.8	19.0	18.5	20.2
1	18.7	19.4	18.9	18.2	20.1
5	15.3	8.5	13.5	12.5	19.7
7	8.4	2.9	10.3	8.0	--
9	4.4	1.1	5.8	3.2	19.1
10	2.6	0.3	4.5	1.4	17.9
12	1.0	--	3.0	0.2	--
15	--	--	1.2	--	17.0

Table 22. Propanol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Propanol Concentration, mg/l				
	Propanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	19.8	21.3	19.5	20.1	19.0
2	15.8	17.3	14.8	16.7	19.0
4	12.2	15.2	13.5	14.2	19.0
6	9.8	11.5	11.6	11.1	19.0
10	4.5	2.6	7.4	4.2	18.0
14	1.5	0.1	4.7	1.4	16.6
18	0.2	--	2.2	--	15.0
20	--	--	1.2	--	--
22	--	--	0.3	--	--

Table 23. 1-Butanol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	1-Butanol Concentration, mg/l				
	1-Butanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	19.9	19.7	18.3	20.3	20.0
4	14.9	13.4	13.0	15.7	18.7
7	9.2	9.6	8.2	11.1	19.2
11	5.0	4.3	5.3	6.1	18.0
13	2.7	1.4	3.9	3.3	--
15	1.8	0.2	2.6	1.6	13.7
17	0.8	--	1.1	--	--

Table 24. Pentanol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Pentanol Concentration, mg/l				
	Pentanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	17.2	18.3	18.4	18.3	17.0
4	13.1	11.0	14.7	12.2	17.0
7	8.3	6.5	9.1	5.5	16.6
11	4.3	2.1	4.4	2.3	--
13	2.8	1.0	3.3	1.2	16.6
15	2.2	0.4	2.4	--	--
17	1.2	--	1.4	--	16.0

Table 25. Phenol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Phenol Concentration, mg/l				
	Phenol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	12.2	11.7	12.2	12.5	14.7
3	10.1	2.9	11.2	10.4	14.1
7	6.0	0.1	8.6	5.2	13.9
10	3.0	--	4.9	0.3	12.4
12	0.5	--	3.6	--	12.0
18	--	--	0.1	--	--

Table 26. Phenol biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	Phenol mg/l	Time (days)	Phenol mg/l	Time (days)	Phenol mg/l	Time (days)	Phenol mg/l
0	192.9	0	56.7	0	4.86	0	0.77
9	145.6	0.8	51.0	0.8	3.37	0.8	0.51
30	97.8	1.4	47.6	1.1	0.93	1.1	0.22
43	32.7	2.0	45.4	1.4	0.60		
48	10.0	4.8	36.1	2.0	0.20		
		7.0	29.1				
		7.8	26.5				
		8.7	23.8				
		9.8	20.9				
		11.8	16.1				
		12.8	13.4				
		13.9	11.3				
		14.2	9.9				
		16.0	5.9				
		18.8	1.9				

Table 27. DCP biodegradation in Blacksburg soil (site 1, 15 feet).

Time (days)	DCP Concentration, mg/l				
	DCP	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	15.8	14.0	12.6	14.1	13.9
7	11.1	5.8	12.4	11.5	13.9
10	10.1	0.7	11.0	9.6	13.7
18	2.1	--	7.3	2.3	13.6

Table 28. Methanol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	Methanol Concentration, mg/l			
	Methanol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	24.0	26.5	26.9	26.9
3	21.5	22.3	20.8	22.2
8	17.0	16.9	8.6	22.3
11	12.2	12.7	3.9	17.8
18	4.0	4.3	--	13.1

Table 29. Ethanol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	Ethanol Concentration, mg/l			
	Ethanol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	17.8	18.0	17.6	20.3
2.8	15.7	17.3	12.5	20.3
5.6	6.8	9.4	1.4	10.5
7.7	2.3	4.1	--	3.4

Table 30. Propanol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	Propanol Concentration, mg/l			
	Propanol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	17.8	17.7	18.1	19.1
2.7	15.5	15.3	13.6	18.0
3.8	12.0	12.8	9.2	15.3
7.7	5.6	5.4	0.4	5.8
9.2	1.9	1.9	--	2.0

Table 31. 1-Butanol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	1-Butanol Concentration, mg/l			
	1-Butanol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	17.8	18.4	18.0	18.4
3	10.4	13.2	11.6	16.3
5	6.6	8.3	6.0	11.2
8	2.7	4.8	1.8	10.3

Table 32. Pentanol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	Pentanol Concentration, mg/l			
	Pentanol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	18.1	24.7	27.2	16.3
2	16.6	21.2	23.0	15.7
5	9.1	12.7	9.3	8.1
8	5.5	7.6	1.9	3.0
12	2.1	3.3	--	0.7

Table 33. Phenol biodegradation in Blacksburg soil (site 2, 4 feet).

Time (days)	Phenol Concentration, mg/l			
	Phenol	+ MoO_4^{-2}	+ NO_3^- + MoO_4^{-2}	+ Mo
0	17.6	18.7	18.3	19.5
2	16.7	16.7	16.0	18.1
5	11.6	12.4	7.9	16.9
8	5.9	6.3	0.1	8.3
12	1.7	0.6	--	0.8

Appendix B
Newport News Biodegradation Data

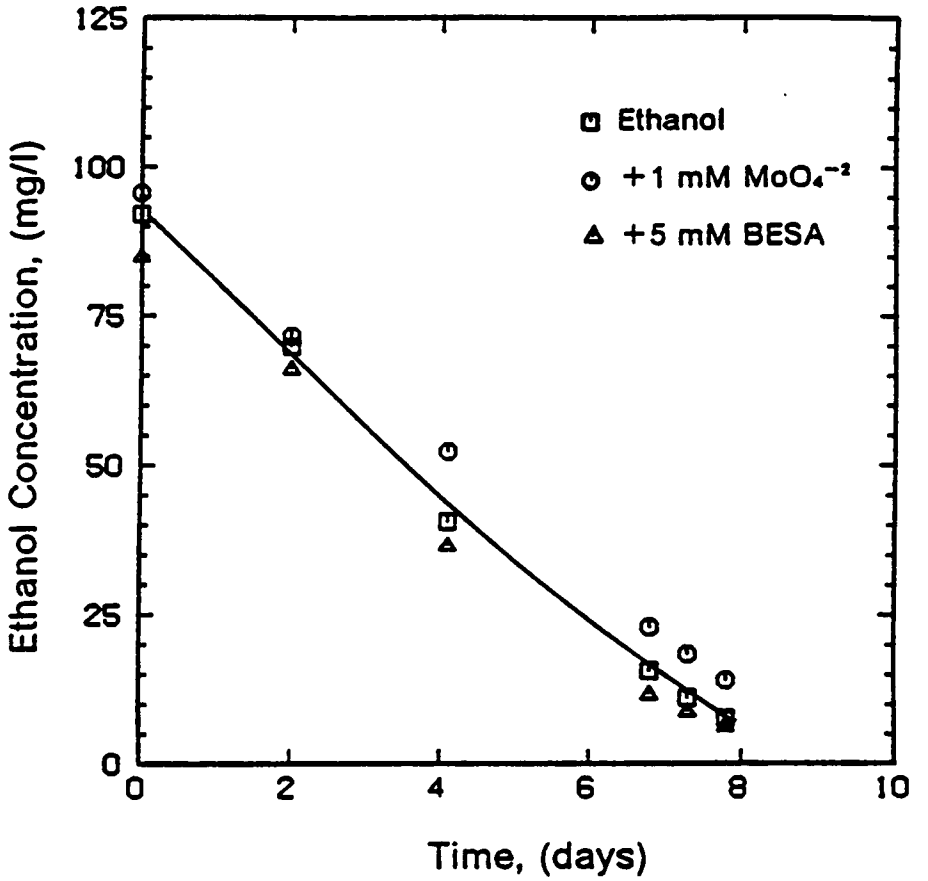

Figure 69. Ethanol biodegradation with molybdate and BESA in Newport News soil.

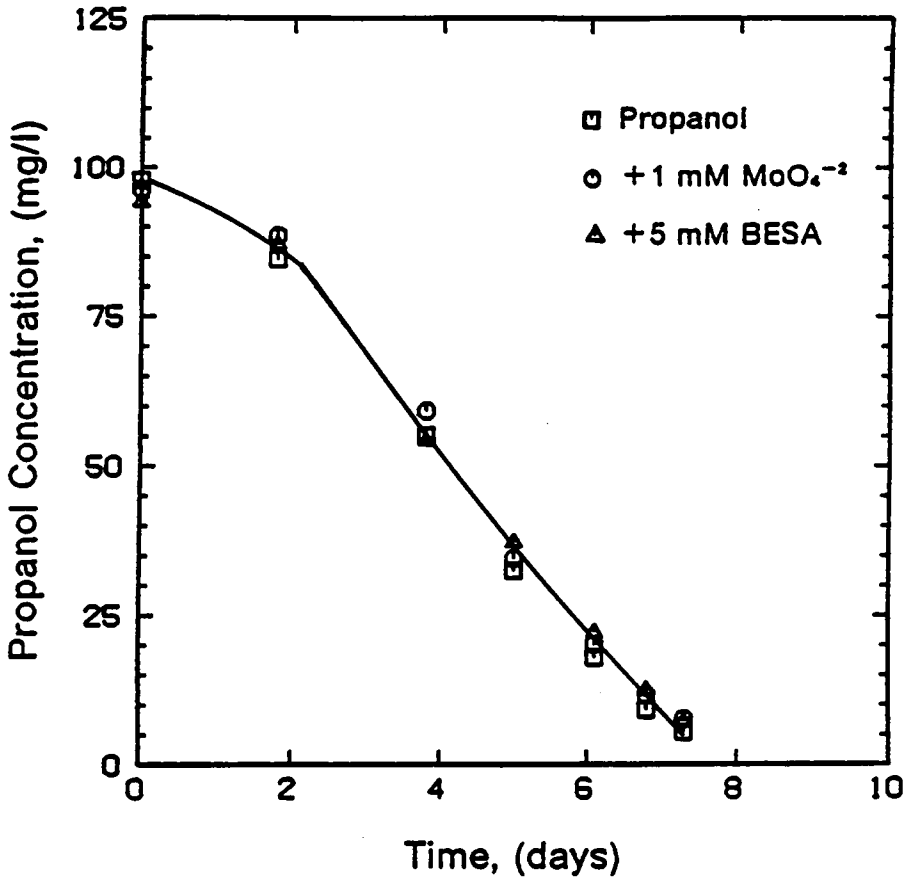

Figure 70. Propanol biodegradation with molybdate and BESA in Newport News soil.

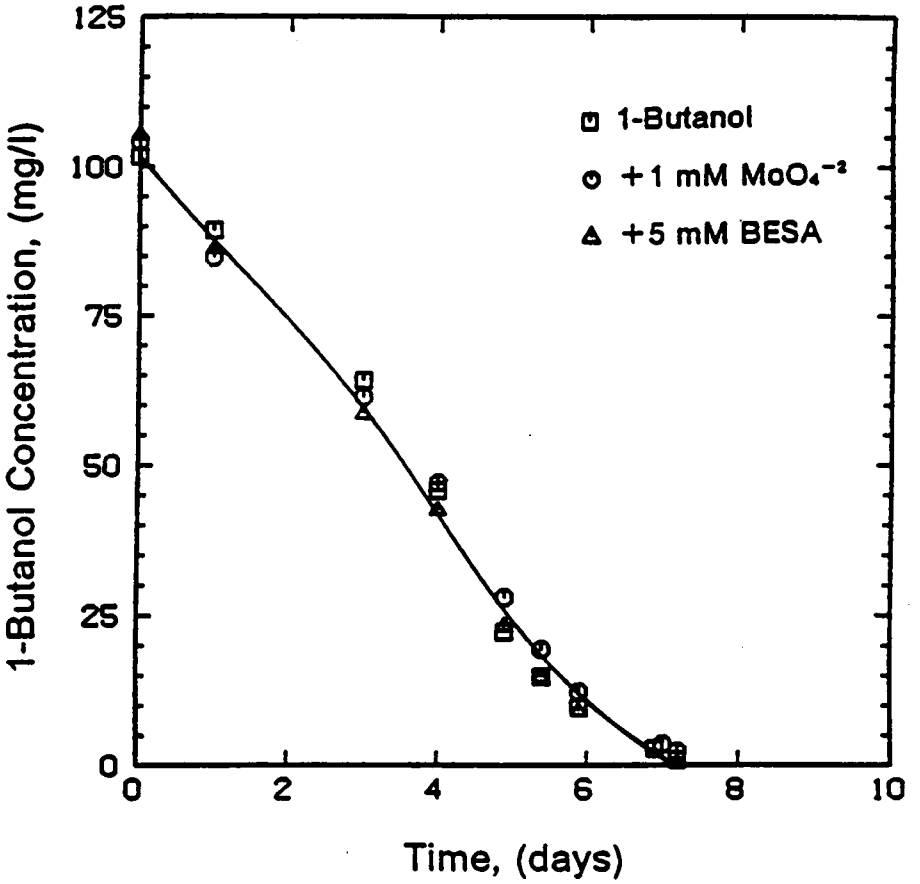

Figure 71. 1-Butanol biodegradation with molybdate and BESA in Newport News soil.

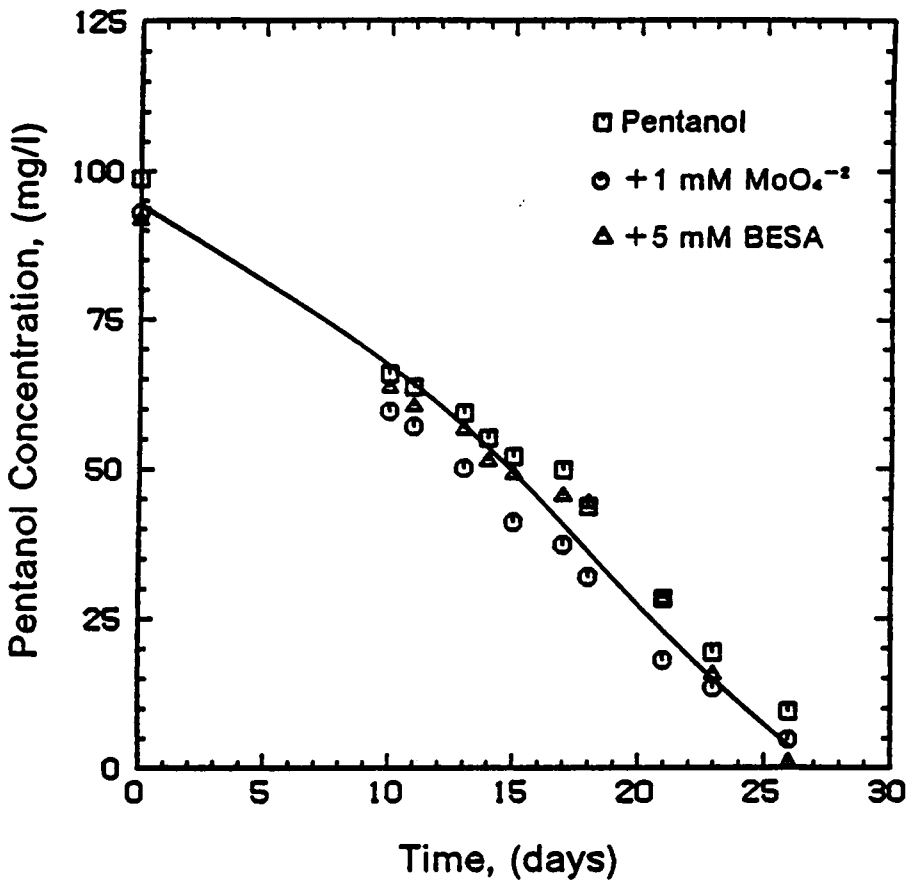

Figure 72. Pentanol biodegradation with molybdate and BESA in Newport News soil.

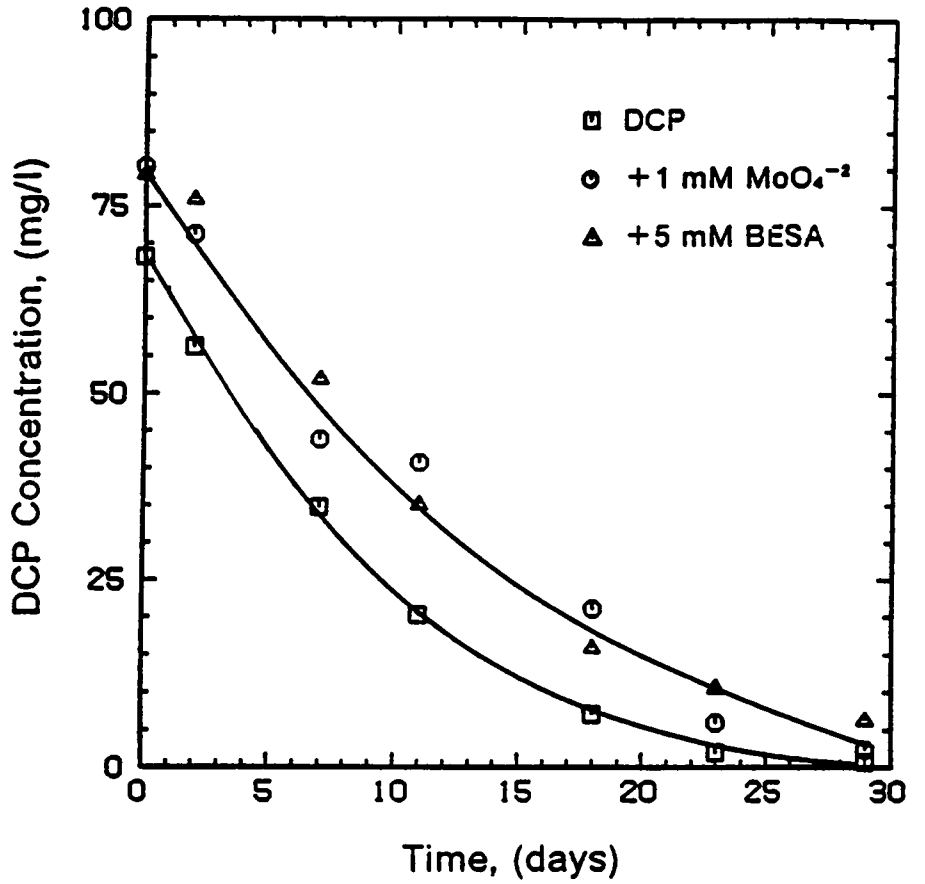

Figure 73. DCP biodegradation with molybdate and BESA in Newport News soil.

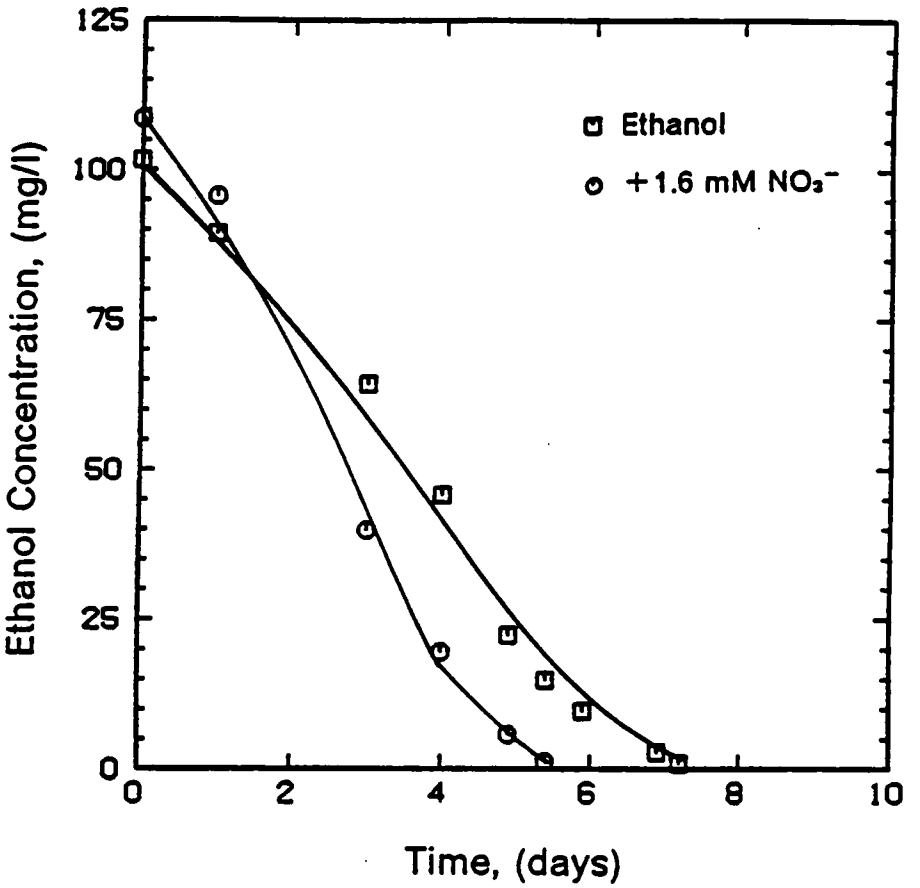

Figure 74. Ethanol biodegradation with nitrate in Newport News soil.

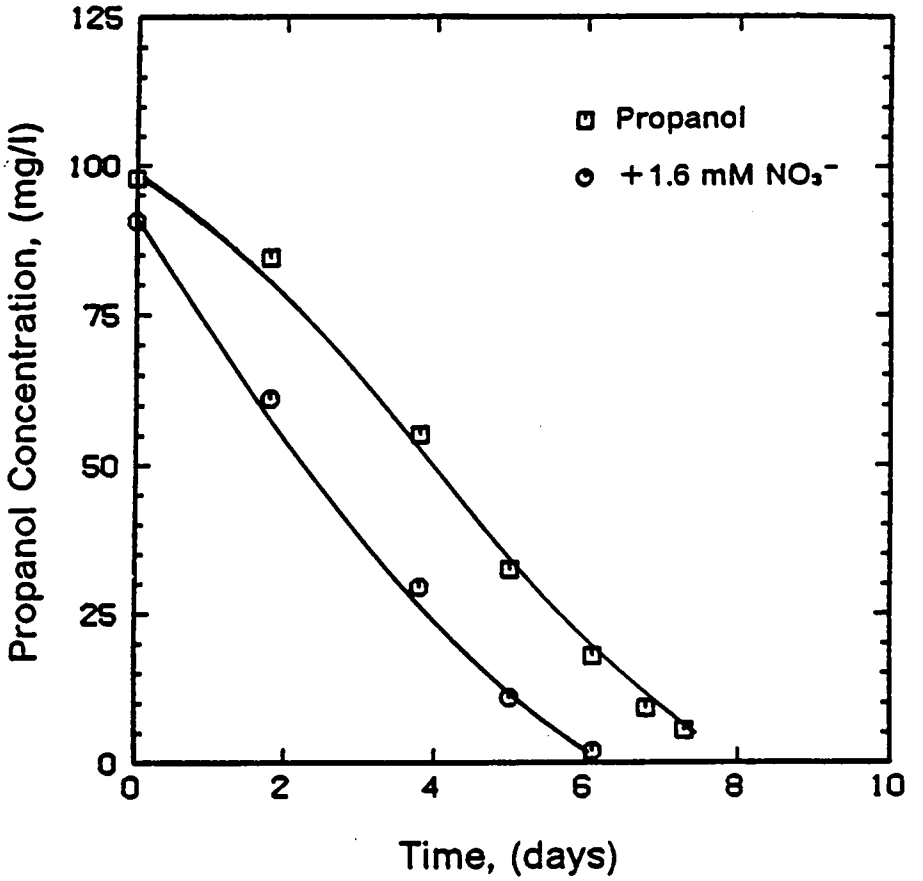

Figure 75. Propanol biodegradation with nitrate in Newport News soil.

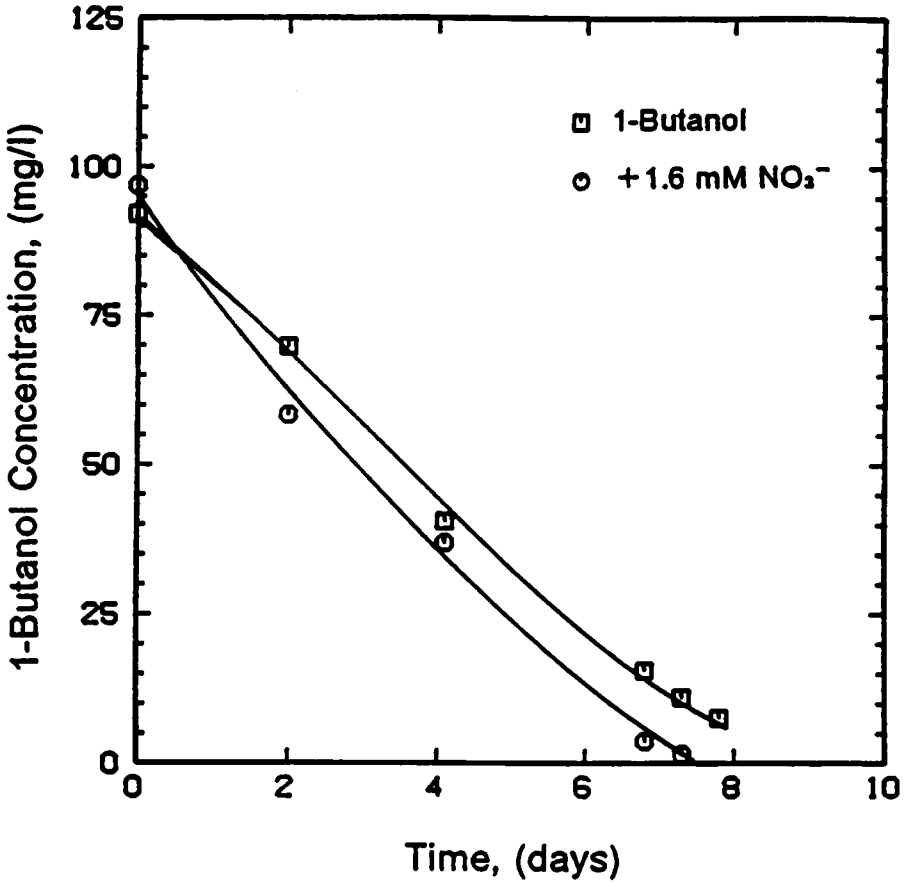

Figure 76. 1-Butanol biodegradation with nitrate in Newport News soil.

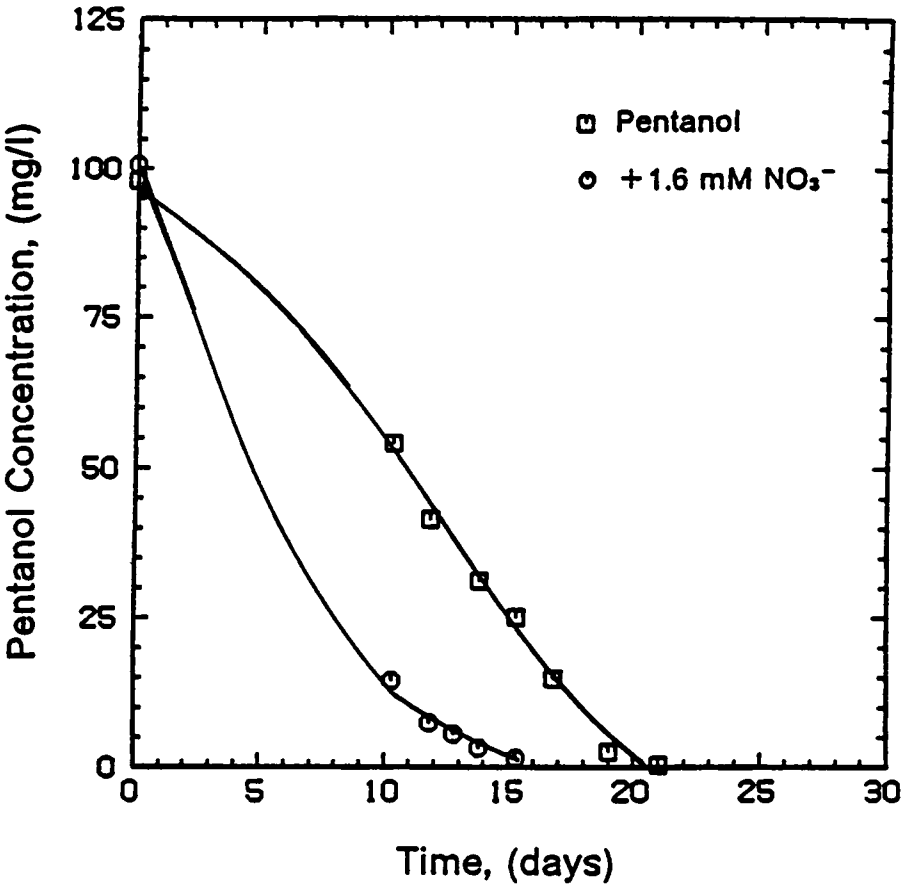

Figure 77. Pentanol biodegradation with nitrate in Newport News soil.

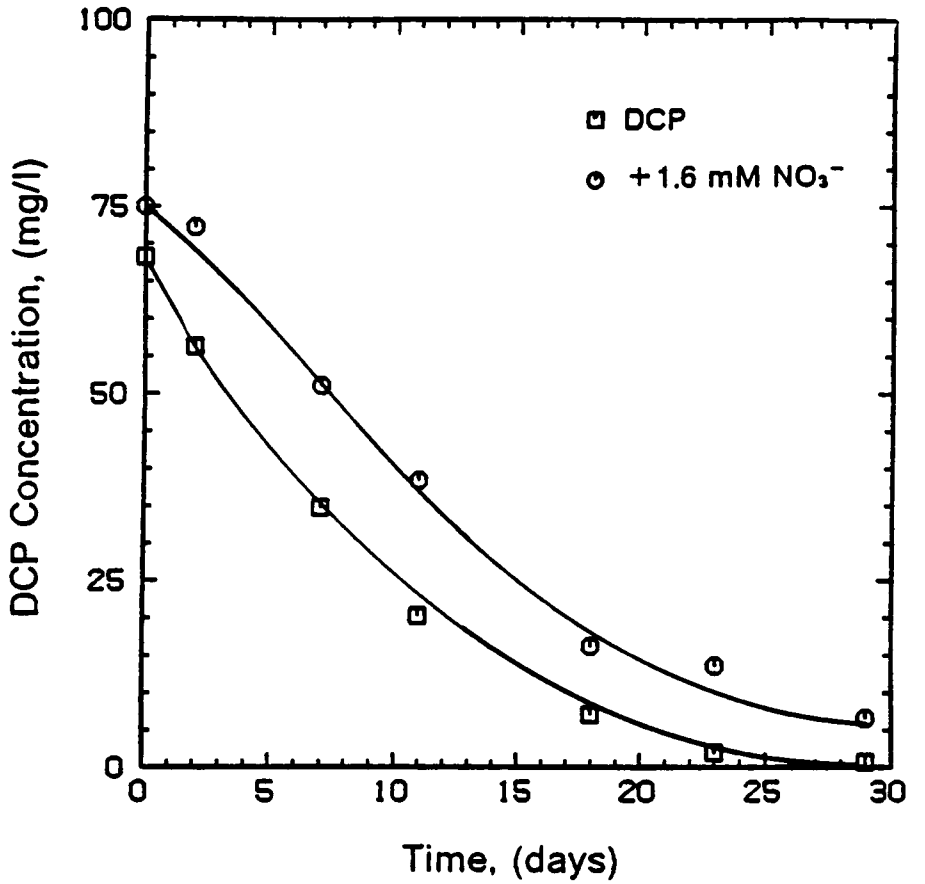

Figure 78. DCP biodegradation with nitrate in Newport News soil.

Table 34. TBA biodegradation in Newport News soil.

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	6330.0	6163.4	6251.5	6081.2
7	6023.9	5798.5	5526.2	6081.5
21	5523.7	5328.5	4657.2	5913.0
44	5342.9	5100.7	4206.8	----
154	4800.0	4702.2	3593.7	----
188	4703.0	----	3449.3	5858.6
261	----	4415.7	3338.9	----
333	4496.3	4360.4	3176.0	5968.0

Table 35. TBA biodegradation in Newport News soil.

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	1226.6	1249.4	1125.3	1110.0
7	1172.4	1180.3	1058.0	1115.5
21	1119.1	1099.0	967.2	1100.0
44	1033.1	1007.2	917.9	----
154	878.2	869.4	585.8	1066.8
188	825.4	818.7	503.8	----
261	769.8	705.3	335.0	----
333	670.0	619.2	101.5	1040.9

Table 36. TBA biodegradation in Newport News soil.

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	261.5	259.8	244.6	241.2
7	241.9	233.3	203.5	239.0
21	229.1	214.3	176.3	246.2
44	182.7	148.7	62.9	----
48	---	---	50.0	----
154	101.7	48.5	---	214.9
188	79.9	23.8	---	212.9
261	20.0	----	----	----

Table 37. TBA biodegradation in Newport News soil.

Time (days)	TBA Concentration, mg/l		
	TBA	+ MoO_4^{-2}	+ NO_3^-
0	95.9	92.5	92.5
10	95.3	89.2	87.8
21	74.3	67.5	67.6
26	71.4	63.8	60.3
32	60.8	54.1	43.0
46	43.7	32.6	4.6
53	33.9	21.0	--
59	28.0	17.0	--
82	3.4	--	--

Table 38. TBA biodegradation in Newport News soil.

Time (days)	TBA Concentration, mg/l			
	TBA	+ MoO_4^{-2}	+ NO_3^-	Autoclaved Control
0	11.8	--	--	--
4	11.4	--	--	--
7	10.5	--	--	--
10	10.2	--	--	--
15	4.8	--	--	--
16	3.5	--	--	--
19	0.2	--	--	--

Table 39. Methanol and TBA biodegradation in Newport News soil.

Time (days)	Alcohol Concentration, mg/l					
	Methanol	Methanol + MoO_4^{-2}	TBA	TBA + MoO_4^{-2}	Methanol/TBA	Methanol/TBA + MoO_4^{-2}
0	20.6	19.3	20.2	19.8	22.5/20.6	21.7/19.7
1	18.1	17.4	18.8	17.9	20.6/19.2	17.8/18.3
2	16.4	15.0	18.0	17.7	19.1/19.1	15.3/18.4
3	12.9	12.3	17.7	17.5	16.1/19.1	12.2/18.4
4	9.8	8.4	--	--	8.6/19.0	5.3/18.3
5	8.3	6.2	17.3	17.1	5.4/18.9	1.7/17.9
6	5.6	4.5	16.3	16.6	3.4/18.8	-- /17.8
8	2.6	1.7	13.5	15.2	2.3/18.9	-- / --
14	--	--	8.0	10.5	-- /17.1	-- /14.7
24	--	--	6.1	5.6	-- /10.0	-- / 7.9
62	--	--	2.3	0.8	-- / 1.0	-- / 4.6

Table 40. Methanol biodegradation in Newport News soil.

Time (days)	Methanol Concentration, mg/l				
	Methanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	98.8	92.9	92.0	96.6	101.3
10	66.0	59.7	63.8	41.8	97.0
11	63.7	57.2	60.6	36.4	--
13	59.3	50.2	56.8	27.0	--
14	55.2	--	51.4	22.3	--
15	52.1	41.1	49.2	17.9	--
17	49.8	37.4	45.6	11.8	97.0
18	43.8	32.0	44.5	8.2	--
21	28.4	18.2	28.5	0.6	--
23	19.3	13.5	15.9	--	--
26	9.6	4.8	1.2	--	97.0

Table 41. Ethanol biodegradation in Newport News soil.

Time (days)	Ethanol Concentration, mg/l				
	Ethanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	101.7	104.2	105.5	108.6	98.7
1	89.3	84.8	86.5	95.7	--
3	64.2	61.4	58.7	39.9	--
4	45.8	47.1	42.7	19.6	--
4.9	22.3	28.0	23.4	5.8	96.0
5.4	14.9	19.4	14.9	1.2	--
5.9	9.7	12.3	9.2	--	--
6.9	2.8	5.7	2.8	--	--
7.0	1.7	3.6	1.2	--	--
7.2	1.0	2.4	0.6	--	95.0

Table 42. Propanol biodegradation in Newport News soil.

Time (days)	Propanol Concentration, mg/l				
	Propanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	97.8	96.2	94.3	90.7	98.5
1.8	84.5	88.4	87.2	61.1	--
3.8	55.2	59.2	54.4	29.5	--
5	32.5	34.6	37.4	10.9	98.5
6.1	17.9	20.2	22.3	2.1	--
6.8	9.2	11.7	12.6	--	--
7.3	5.4	7.6	7.4	--	98.5

Table 43. 1-Butanol biodegradation in Newport News soil.

Time (days)	1-Butanol Concentration, mg/l				
	1-Butanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	92.1	95.6	85.0	96.8	97.9
2	69.9	71.5	66.2	58.5	--
4.1	40.7	52.3	36.7	37.1	97.0
6.8	15.6	23.0	11.8	3.8	--
7.3	11.1	18.4	8.9	1.6	--
7.8	7.7	14.0	6.5	--	95.9

Table 44. Pentanol biodegradation in Newport News soil.

Time (days)	Pentanol Concentration, mg/l				
	Pentanol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	98.0	90.0	93.2	100.7	104.3
10.3	54.2	47.2	41.5	14.5	98.2
11.8	41.6	36.8	29.3	7.5	--
12.8	--	--	--	5.6	--
13.8	31.3	25.6	21.1	3.3	--
15.3	25.2	20.3	11.4	1.5	89.0
16.8	14.8	14.9	3.9	--	--
19.0	2.6	7.6	--	--	--
21.0	0.4	3.9	--	--	86.0

Table 45. Phenol biodegradation in Newport News soil.

Time (days)	Phenol Concentration, mg/l				
	Phenol	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	100.5	97.3	107.8	104.2	103.7
2	97.3	91.2	100.7	97.5	--
7	74.7	73.1	88.1	82.1	--
14	49.5	43.6	67.0	51.0	97.3
18	29.9	33.1	56.1	39.3	94.2
23	18.7	22.7	49.3	21.4	--
29	5.1	5.4	29.8	7.7	93.1

Table 46. DCP biodegradation in Newport News soil.

Time (days)	DCP Concentration, mg/l				
	DCP	+ MoO_4^{-2}	+ BESA	+ NO_3^-	Autoclaved Control
0	68.2	80.2	79.3	75.0	80.9
2	56.3	71.2	76.0	72.2	--
7	34.7	43.9	52.0	51.0	81.0
11	20.3	40.8	35.2	38.4	--
18	7.1	21.1	16.0	16.2	--
23	2.0	6.0	10.7	13.6	53.2
29	0.8	2.4	6.4	6.6	--

Appendix C
Thermodynamic Calculations

Methanol

$CH_3OH + H_2O = CO_2 + 6H^+ + 6e^-$

$\Delta G° = -94.26 - (-39.87 - 56.69)$

$\Delta G° = 2.30$ kcal

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$K = 10^{-\Delta G°/2.3RT}$

$K = 10^{-1.687}$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$-\Delta G_r(W) = pe(2.3nRT)$

$\Delta G_r(W) = -9.151$ kcal/electron equivalent

$\frac{1}{6}CH_3OH + \frac{1}{6}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -9.15$ kcal/mole

$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O$ $\Delta G_r(W) = -18.68$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{4}O_2 = \frac{1}{6}CO_2 + \frac{1}{3}H_2O$ $\Delta G_r(W) = -27.83$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{6}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -9.15$ kcal/mole

$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O$ $\Delta G_r(W) = -17.13$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{1}{6}CO_2 + \frac{1}{10}N_2 + + \frac{13}{30}H_2O$ $\Delta G_r(W) = -26.28$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{6}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -9.15$ kcal/mole

$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O$ $\Delta G_r(W) = 5.09$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O$ $\Delta G_r(W) = -4.07$ kcal/mole

$\frac{1}{6}CH_3OH + \frac{1}{6}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -9.15$ kcal/mole

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{6}CH_3OH = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 + \frac{1}{12}H_2O \qquad \Delta G_r(W) = -3.39 \text{ kcal/mole}$$

Ethanol

$$CH_3CH_2OH + 3H_2O = 2CO_2 + 12H^+ + 12e^-$$

$$\Delta G° = 2(-94.26) - (-41.63 - 3(56.69))$$

$$\Delta G° = 23.17 \text{ kcal}$$

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$$K = 10^{-\Delta G°/2.3RT}$$

$$K = 10^{-1.687}$$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$$-\Delta G_r(W) = pe(2.3nRT)$$

$$\Delta G_r(W) = -7.61 \text{ kcal/electron equivalent}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{4}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.61 \text{ kcal/mole}$$

$$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O \quad \Delta G_r(W) = -18.68 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{4}O_2 = \frac{1}{6}CO_2 + \frac{1}{4}H_2O \quad \Delta G_r(W) = -26.28 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{4}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.61 \text{ kcal/mole}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O \quad \Delta G_r(W) = -17.13 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{5}NO_3^- + \frac{1}{H^+} = \frac{1}{6}CO_2 + \frac{1}{10}N_2 + \frac{7}{20}H_2O \quad \Delta G_r(W) = -24.74 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{4}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.61 \text{ kcal/mole}$$

$$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O \quad \Delta G_r(W) = 5.09 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{4}H_2O \quad \Delta G_r(W) = -2.52 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH + \frac{1}{4}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.61 \text{ kcal/mole}$$

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{12}CH_3CH_2OH = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 \qquad \Delta G_r(W) = -1.84 \text{ kcal/mole}$$

Propanol

$CH_3(CH_2)_2OH + 5H_2O = 3CO_2 + 18H^+ + 18e^-$

$\Delta G° = -3(94.26) - (-40.78 - 5(56.69))$

$\Delta G° = 41.44$ kcal

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$K = 10^{-\Delta G°/2.3RT}$

$K = 10^{-1.687}$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$-\Delta G_r(W) = pe(2.3nRT)$

$\Delta G_r(W) = -7.24$ kcal/electron equivalent

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{5}{18}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.24$ kcal/mole

$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O$ $\Delta G_r(W) = -18.68$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{1}{4}O_2 = \frac{2}{9}H_2O + \frac{1}{6}CO_2$ $\Delta G_r(W) = -25.91$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{5}{18}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.24$ kcal/mole

$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O$ $\Delta G_r(W) = -17.13$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{1}{6}CO_2\frac{1}{10}N_2 + \frac{29}{90}H_2O$ $\Delta G_r(W) = -24.36$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{5}{18}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.24$ kcal/mole

$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O$ $\Delta G_r(W) = 5.09$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2 + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{2}{9}H_2O$ $\Delta G_r(W) = -2.15$ kcal/mole

$\frac{1}{18}CH_3(CH_2)_2OH + \frac{5}{18}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.24$ kcal/mole

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{18}CH_3(CH_2)_2OH + \frac{1}{36}H_2O = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 \quad \Delta G_r(W) = -1.47 \text{ kcal/mole}$$

1-Butanol

$$CH_3(CH_2)_3OH + 7H_2O = 4CO_2 + 24H^+ + 24e^-$$

$$\Delta G° = -4(94.26) - (-38.84 - 7(56.69))$$

$$\Delta G° = 58.61 \text{ kcal}$$

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$$K = 10^{-\Delta G°/2.3RT}$$

$$K = 10^{-1.687}$$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$$-\Delta G_r(W) = pe(2.3nRT)$$

$$\Delta G_r(W) = -7.10 \text{ kcal/electron equivalent}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.10 \text{ kcal/mole}$$

$$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O \quad \Delta G_r(W) = -18.68 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{1}{4}O_2 = \frac{1}{6}CO_2 + \frac{5}{24}H_2O \quad \Delta G_r(W) = -25.77 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.10 \text{ kcal/mole}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O \quad \Delta G_r(W) = -17.13 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{1}{6}CO_2 + \frac{1}{10}N_2 + \frac{37}{120}H_2O \quad \Delta G_r(W) = -24.22 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.10 \text{ kcal/mole}$$

$$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O \quad \Delta G_r(W) = 5.09 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{5}{24}H_2O \quad \Delta G_r(W) = -2.01 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -7.10 \text{ kcal/mole}$$

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{24}CH_3(CH_2)_3OH + \frac{1}{24}H_2O = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 \qquad \Delta G_r(W) = -1.33 \text{ kcal/mole}$$

Pentanol

$$CH_3(CH_2)_4OH + 9H_2O = 5CO_2 + 30H^+ + 30e^-$$

$$\Delta G^\circ = -5(94.26) - (-38.30 - 9(56.69))$$

$$\Delta G^\circ = 77.18 \text{ kcal}$$

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$$K = 10^{-\Delta G^\circ/2.3RT}$$

$$K = 10^{-1.687}$$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$$-\Delta G_r(W) = pe(2.3nRT)$$

$$\Delta G_r(W) = -6.97 \text{ kcal/electron equivalent}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{9}{30}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -6.97 \text{ kcal/mole}$$

$$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O \quad \Delta G_r(W) = -18.68 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{1}{4}O_2 = \frac{1}{6}CO_2 + \frac{1}{5}H_2O \quad \Delta G_r(W) = -25.64 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{9}{30}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -6.97 \text{ kcal/mole}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O \quad \Delta G_r(W) = -17.13 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{1}{6}CO_2 + \frac{1}{10}N_2 + \frac{3}{10}H_2O \quad \Delta G_r(W) = -24.09 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{9}{30}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -6.97 \text{ kcal/mole}$$

$$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O \quad \Delta G_r(W) = 5.09 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{5}H_2O \quad \Delta G_r(W) = -1.88 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{9}{30}H_2O = \frac{1}{6}CO_2 + H^+ + e^- \quad \Delta G_r(W) = -6.97 \text{ kcal/mole}$$

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{30}CH_3(CH_2)_4OH + \frac{1}{20}H_2O = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 \quad \Delta G_r(W) = -1.20 \text{ kcal/mole}$$

TBA

$C(CH_3)_3OH + 7H_2O = 4CO_2 + 24H^+ + 24e^-$

$\Delta G° = -4(94.26) - (-44.14 - 7(56.69))$

$\Delta G° = 63.91$ kcal

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$K = 10^{-\Delta G°/2.3RT}$

$K = 10^{-1.687}$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$-\Delta G_r(W) = pe(2.3nRT)$

$\Delta G_r(W) = -6.88$ kcal/electronequivalent

$\frac{1}{24}C(CH_3)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -25.55$ kcal/mole

$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O$ $\Delta G_r(W) = -18.68$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{1}{4}O_2 = \frac{1}{6}CO_2 + \frac{5}{24}H_2O$ $\Delta G_r(W) = -25.55$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -25.55$ kcal/mole

$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O$ $\Delta G_r(W) = -17.13$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{1}{6}CO_2 + \frac{1}{10}N_2 + \frac{37}{120}H_2O$ $\Delta G_r(W) = -24.00$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -25.55$ kcal/mole

$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O$ $\Delta G_r(W) = 5.09$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{1}{6}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{5}{24}H_2O$ $\Delta G_r(W) = -1.79$ kcal/mole

$\frac{1}{24}C(CH_3)_3OH + \frac{7}{24}H_2O = \frac{1}{6}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -25.55$ kcal/mole

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{24}C(CH_3)_3OH + \frac{1}{24}H_2O = \frac{1}{24}CO_2 + \frac{1}{8}CH_4 \qquad \Delta G_r(W) = -1.11 \text{ kcal/mole}$$

Phenol

$C_6H_5OH + 11H_2O = 6CO_2 + 28H^+ + 28e^-$

$\Delta G° = 6(-94.26) - (-11.02 - 11(56.69))$

$\Delta G° = 69.02$ kcal

$$pe = \frac{1}{n}\log K + \frac{1}{n}\log\frac{[CH_3OH]}{[CO_2][H^+]^6}$$

$$pe = \frac{1}{6}\log K + \frac{1}{6}\log[CH_3OH] - \frac{1}{6}\log[CO_2] - \log[H^+]$$

$K = 10^{-\Delta G°/2.3RT}$

$K = 10^{-1.687}$

$$pe = \frac{1}{6}(\log 10^{-1.687}) + pH$$

$-\Delta G_r(W) = pe(2.3nRT)$

$\Delta G_r(W) = -7.07$ kcal/electron equivalent

$\frac{1}{28}C_6H_5OH + \frac{11}{28}H_2O = \frac{3}{14}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.07$ kcal/mole

$\frac{1}{4}O_2 + H^+ + e^- = \frac{1}{2}H_2O$ $\Delta G_r(W) = -18.68$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{1}{4}O_2 = \frac{3}{14}CO_2 + \frac{3}{28}H_2O$ $\Delta G_r(W) = -25.75$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{11}{28}H_2O = \frac{3}{14}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.07$ kcal/mole

$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- = \frac{1}{10}N_2 + \frac{3}{5}H_2O$ $\Delta G_r(W) = -17.13$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ = \frac{3}{14}CO_2 + \frac{1}{10}N_2 + \frac{29}{140}H_2O$ $\Delta G_r(W) = -24.20$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{11}{28}H_2O = \frac{3}{14}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.07$ kcal/mole

$\frac{1}{8}SO_4^{-2} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{3}H_2O$ $\Delta G_r(W) = 5.09$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{1}{8}SO_4^{-2} + \frac{3}{16}H^+ = \frac{3}{14}CO_2 + \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{3}{28}H_2O$ $\Delta G_r(W) = -2.00$ kcal/mole

$\frac{1}{28}C_6H_5OH + \frac{11}{28}H_2O = \frac{3}{14}CO_2 + H^+ + e^-$ $\Delta G_r(W) = -7.07$ kcal/mole

$$\frac{1}{8}CO_2 + H^+ + e^- = \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G_r(W) = 5.76 \text{ kcal/mole}$$

$$\frac{1}{28}C_6H_5OH + \frac{1}{7}H_2O = \frac{5}{56}CO_2 + \frac{1}{8}CH_4 \quad \Delta G_r(W) = -1.31 \text{ kcal/mole}$$

The following calculations represent hydrogen-dependent reactions for ethanol, propanol, 1-butanol and pentanol under methanogenic conditions. All reactants and products have been assumed to be at unit activity except the following: $[CH_{4(g)}] = 0.7$ atm; $[CO_{2(g)}] = 0.3$ atm; pH = 7.0; T = 298 °K. Free energy values for all compounds were obtained from Lang's Handbook of Chemistry (1972).

Ethanol

1. Acetogenesis

$$CH_3CH_2OH_{(aq)} + H_2O_{(l)} = CH_3COO^-_{(aq)} + H^+_{(aq)} + 2H_{2(g)}$$

$$\Delta G° = -88.29 - (-41.63 + (-56.69)) = 10.03 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[H_{2(g)}]^2[H^+_{(aq)}][CH_3COO^-_{(aq)}]}{[CH_3CH_2OH_{(aq)}]}$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CH_3COO^-_{(aq)}] - \ln[CH_3CH_2OH_{(aq)}])$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] + \ln[H^+_{(aq)}])$$

$$\Delta G = 10.03 + 0.592(2\ln[H_{2(g)}] - 16.12)$$

2. Proton Reduction

$$2H_{2(g)} + \frac{1}{2}CO_{2(g)} = \frac{1}{2}CH_{4(g)} + H_2O_{(l)}$$

$$\Delta G° = -56.69 + (\tfrac{1}{2})(-12.15) - (\tfrac{1}{2})(-94.26) = -15.64 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}]^{\frac{1}{2}}}{[H_{2(g)}]^2[CO_{2(g)}]^{\frac{1}{2}}}$$

$$\Delta G = -15.64 + 0.592(\tfrac{1}{2}\ln[CH_{4(g)}] - 2\ln[H_{2(g)}] - \tfrac{1}{2}\ln[CO_{2(g)}])$$

$$\Delta G = -15.64 + 0.592(\tfrac{1}{2}\ln(0.7) - 2\ln[H_{2(g)}] - \tfrac{1}{2}\ln(0.3))$$

$$\Delta G = -15.64 + 0.592(0.424 - 2\ln[H_{2(g)}])$$

3. Methane and Carbon Dioxide Production from Acetate

$$CH_3COO^-_{(aq)} + H^+_{(aq)} = CH_{4(g)} + CO_{2(g)}$$

$$\Delta G° = -12.15 + (-94.26) - (-88.29) = -18.12 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}][CO_{2(g)}]}{[CH_3COO^-_{(aq)}][H^+_{(aq)}]}$$

$$\Delta G = -18.19 + 0.592(\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3COO^-_{(aq)}] - \ln[H^+_{(aq)}])$$

$$\Delta G = -18.19 + 0.592(\ln(0.7) + \ln(0.3) - \ln(10^{-7}))$$

$$\Delta G = -9.50 \text{ kcal}$$

4. Net Reaction

$$CH_3CH_2OH_{(aq)} = \frac{3}{2}CH_{4(g)} + \frac{1}{2}CO_2(g)$$

$$\Delta G^\circ = \frac{1}{2}(-94.26) + \frac{3}{2}(-12.15) - (-41.63) = -23.73 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^{\frac{3}{2}}[CO_{2(g)}]^{\frac{1}{2}}}{[CH_3CH_2OH_{(aq)}]}$$

$$\Delta G = -23.73 + 0.592(\frac{3}{2}\ln[CH_{4(g)}] + \frac{1}{2}\ln[CO_{2(g)}] - \ln[CH_3CH_2OH])$$

$$\Delta G = -23.73 + 0.592(\frac{3}{2}\ln(0.7) + \frac{1}{2}\ln(0.3))$$

$$\Delta G = -24.40 \text{ kcal}$$

Propanol

1. Acetogenesis

$$CH_3(CH_2)_2OH_{(aq)} + 3H_2O_{(l)} = CH_3COO^-_{(aq)} + H^+_{(aq)} + 5H_{2(g)} + CO_{2(g)}$$

$$\Delta G^\circ = -88.29 + (-94.26) - (-40.78 + 3(-56.69)) = 28.30 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[H_{2(g)}]^5[H^+_{(aq)}][CH_3COO^-_{(aq)}][CO_{2(g)}]}{[CH_3(CH_2)_2OH_{(aq)}]}$$

$$\Delta G = 28.30 + 0.592(5\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CH_3COO^-_{(aq)}] + \ln[CO_{2(g)}] - \ln[CH_3(CH_2)_2OH_{(aq)}])$$

$$\Delta G = 28.30 + 0.592(5\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CO_{2(g)}])$$

$$\Delta G = 28.30 + 0.592(5\ln[H_{2(g)}] + \ln(10^{-7}) + \ln(0.3))$$

$$\Delta G = 28.30 + 0.592(5\ln[H_{2(g)}] - 17.32)$$

2. Proton Reduction

$$5H_{2(g)} + \frac{5}{4}CO_{2(g)} = \frac{5}{4}CH_{4(g)} + \frac{5}{2}H_2O_{(l)}$$

$$\Delta G^\circ = (\frac{5}{2})(-56.69) + (\frac{5}{4})(-12.15) - (\frac{5}{4})(-94.26) = -39.09 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^{\frac{5}{4}}}{[H_{2(g)}]^5[CO_{2(g)}]^{\frac{5}{4}}}$$

$$\Delta G = -39.09 + 0.592(\frac{5}{4}\ln[CH_{4(g)}] - 5\ln[H_{2(g)}] - \frac{5}{4}\ln[CO_{2(g)}])$$

$$\Delta G = -39.09 + 0.592(\frac{5}{4}\ln(0.7) - 5\ln[H_{2(g)}] - \frac{5}{4}\ln(0.3))$$

$$\Delta G = -39.09 + 0.592(1.06 - 5\ln[H_{2(g)}])$$

3. Methane and Carbon Dioxide Production from Acetate

$$CH_3COO^-_{(aq)} + H^+_{(aq)} = CH_{4(g)} + CO_{2(g)}$$

$$\Delta G^\circ = -12.15 + (-94.26) - (-88.29) = -18.12 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}][CO_{2(g)}]}{[CH_3COO^-_{(aq)}][H^+_{(aq)}]}$$

$$\Delta G = -18.19 + 0.592(\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3COO^-_{(aq)}] - \ln[H^+_{(aq)}])$$

$$\Delta G = -18.19 + 0.592(\ln(0.7) + \ln(0.3) - \ln(10^{-7}))$$

$$\Delta G = -9.50 \text{ kcal}$$

4. Net Reaction

$$CH_3(CH_2)_2OH_{(aq)} + \frac{1}{2}H_2O_{(l)} = \frac{9}{4}CH_{4(g)} + \frac{3}{4}CO_2(g)$$

$$\Delta G^\circ = (\frac{3}{4})(-94.26) + (\frac{9}{4})(-12.15) - (\frac{1}{2})(-56.69) - (40.78) = -28.91 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^{\frac{9}{4}}[CO_{2(g)}]^{\frac{3}{4}}}{[CH_3(CH_2)_2OH_{(aq)}]}$$

$$\Delta G = -28.91 + 0.592(\frac{9}{4}\ln[CH_{4(g)}] + \frac{3}{4}\ln[CO_{2(g)}] - \ln[CH_3(CH_2)_2OH])$$

$$\Delta G = -28.91 + 0.592(\frac{9}{4}\ln(0.7) + \frac{3}{4}\ln(0.3))$$

$$\Delta G = -29.92 \text{ kcal}$$

1-Butanol

1. Acetogenesis

$$CH_3(CH_2)_3OH_{(aq)} + 5H_2O_{(l)} = CH_3COO^-_{(aq)} + H^+_{(aq)} + 8H_{2(g)} + 2CO_{2(g)}$$

$$\Delta G° = -88.29 + 2(-94.26) - (-38.84 + 5(-56.69)) = 45.45 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[H_{2(g)}]^8[H^+_{(aq)}][CH_3COO^-_{(aq)}][CO_{2(g)}]^2}{[CH_3(CH_2)_3OH_{(aq)}]}$$

$$\Delta G = 45.45 + 0.592(8\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CH_3COO^-_{(aq)}] + 2\ln[CO_{2(g)}] - \ln[CH_3(CH_2)_3OH_{(aq)}])$$

$$\Delta G = 45.45 + 0.592(8\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + 2\ln[CO_{2(g)}])$$

$$\Delta G = 45.45 + 0.592(8\ln[H_{2(g)}] + \ln(10^{-7}) + 2\ln(0.3))$$

$$\Delta G = 45.45 + 0.592(8\ln[H_{2(g)}] - 18.53)$$

2. Proton Reduction

$$8H_{2(g)} + 2CO_{2(g)} = 2CH_{4(g)} + 4H_2O_{(l)}$$

$$\Delta G° = 4(-56.69) + 2(-12.15) - 2(-94.26) = -62.54 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}]^2}{[H_{2(g)}]^8[CO_{2(g)}]^2}$$

$$\Delta G = -62.54 + 0.592(2\ln[CH_{4(g)}] - 8\ln[H_{2(g)}] - 2\ln[CO_{2(g)}])$$

$$\Delta G = -62.54 + 0.592(2\ln(0.7) - 8\ln[H_{2(g)}] - 2\ln(0.3))$$

$$\Delta G = -62.54 + 0.592(1.69 - 8\ln[H_{2(g)}])$$

3. Methane and Carbon Dioxide Production from Acetate

$$CH_3COO^-_{(aq)} + H^+_{(aq)} = CH_{4(g)} + CO_{2(g)}$$

$$\Delta G° = -12.15 + (-94.26) - (-88.29) = -18.12 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}][CO_{2(g)}]}{[CH_3COO^-_{(aq)}][H^+_{(aq)}]}$$

$$\Delta G = -18.19 + 0.592(\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3COO^-_{(aq)}] - \ln[H^+_{(aq)}])$$

$$\Delta G = -18.19 + 0.592(\ln(0.7) + \ln(0.3) - \ln(10^{-7}))$$

$$\Delta G = -9.50 \text{ kcal}$$

4. Net Reaction

$$CH_3(CH_2)_3OH_{(aq)} + H_2O_{(l)} = 3CH_{4(g)} + CO_2(g)$$

$$\Delta G° = (-94.26) + 3(-12.15) - (-56.69) - (-38.84) = -35.18 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^3[CO_{2(g)}]}{[CH_3(CH_2)_3OH_{(aq)}]}$$

$$\Delta G = -35.18 + 0.592(3\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3(CH_2)_3OH])$$

$$\Delta G = -35.18 + 0.592(3\ln(0.7) + \ln(0.3))$$

$$\Delta G = -36.53 \text{ kcal}$$

Pentanol

1. Acetogenesis

$$CH_3(CH_2)_4OH_{(aq)} + 7H_2O_{(l)} = CH_3COO^-_{(aq)} + H^+_{(aq)} + 11H_{2(g)} + 3CO_{2(g)}$$

$$\Delta G° = -88.29 + 3(-94.26) - (-38.30 + 7(-56.69)) = 64.06 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[H_{2(g)}]^{11}[H^+_{(aq)}][CH_3COO^-_{(aq)}][CO_{2(g)}]^3}{[CH_3(CH_2)_4OH_{(aq)}]}$$

$$\Delta G = 64.06 + 0.592(11\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + \ln[CH_3COO^-_{(aq)}] + 3\ln[CO_{2(g)}] - \ln[CH_3(CH_2)_4OH_{(aq)}])$$

$$\Delta G = 64.06 + 0.592(11\ln[H_{2(g)}] + \ln[H^+_{(aq)}] + 3\ln[CO_{2(g)}])$$

$$\Delta G = 64.06 + 0.592(11\ln[H_{2(g)}] + \ln(10^{-7}) + 3\ln(0.3))$$

$$\Delta G = 64.06 + 0.592(11\ln[H_{2(g)}] - 19.73)$$

2. Proton Reduction

$$11H_{2(g)} + \frac{11}{4}CO_{2(g)} = \frac{11}{4}CH_{4(g)} + \frac{11}{2}H_2O_{(l)}$$

$$\Delta G° = (\frac{11}{2})(-56.69) + (\frac{11}{4})(-12.15) - (\frac{11}{4})(-94.26) = -85.99 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}]^{\frac{11}{4}}}{[H_{2(g)}]^{11}[CO_{2(g)}]^{\frac{11}{4}}}$$

$$\Delta G = -85.99 + 0.592(\frac{11}{4}\ln[CH_{4(g)}] - 11\ln[H_{2(g)}] - \frac{11}{4}\ln[CO_{2(g)}])$$

$$\Delta G = -85.99 + 0.592(\frac{11}{4}\ln(0.7) - 11\ln[H_{2(g)}] - \frac{11}{4}\ln(0.3))$$

$$\Delta G = -85.99 + 0.592(2.33 - 11\ln[H_{2(g)}])$$

3. Methane and Carbon Dioxide Production from Acetate

$$CH_3COO^-_{(aq)} + H^+_{(aq)} = CH_{4(g)} + CO_{2(g)}$$

$$\Delta G° = -12.15 + (-94.26) - (-88.29) = -18.12 \text{ kcal}$$

$$\Delta G = \Delta G° + RT\ln\frac{[CH_{4(g)}][CO_{2(g)}]}{[CH_3COO^-_{(aq)}][H^+_{(aq)}]}$$

$$\Delta G = -18.19 + 0.592(\ln[CH_{4(g)}] + \ln[CO_{2(g)}] - \ln[CH_3COO^-_{(aq)}] - \ln[H^+_{(aq)}])$$

$$\Delta G = -18.19 + 0.592(\ln(0.7) + \ln(0.3) - \ln(10^{-7}))$$

$$\Delta G = -9.50 \text{ kcal}$$

4. Net Reaction

$$CH_3(CH_2)_4OH_{(aq)} + \frac{3}{2}H_2O_{(l)} = \frac{15}{4}CH_{4(g)} + \frac{5}{4}CO_2(g)$$

$$\Delta G^\circ = (\frac{5}{4})(-94.26) + (\frac{15}{4})(-12.15) - (\frac{3}{2})(-56.69) - (-38.30) = -40.08 \text{ kcal}$$

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_{4(g)}]^{\frac{15}{4}}[CO_{2(g)}]^{\frac{5}{4}}}{[CH_3(CH_2)_4OH_{(aq)}]}$$

$$\Delta G = -40.08 + 0.592(\frac{15}{4}\ln[CH_{4(g)}] + \frac{5}{4}\ln[CO_{2(g)}] - \ln[CH_3(CH_2)_4OH])$$

$$\Delta G = -40.08 + 0.592(\frac{15}{4}\ln(0.7) + \frac{5}{4}\ln(0.3))$$

$$\Delta G = -41.76 \text{ kcal}$$

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